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THE SEROLOGICAL REACTIONS OF VIRUSES CAUSING TOBACCO NECROSIS.

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THE purification of tobacco necrosis virus has been described by Pirie, Smith, Spooner and MacClement (1938). Their method involved precipitating the virus from infective sap by the addition of three volumes of alcohol followed by fractional precipitation with ammonium sulphate, and their end-product always consisted of two fractions. One was a crystalline nucleoprotein, which sedimented in the ultra-centrifuge with a single sharp boundary corresponding to $S_{w20^\circ} = 130 \times 10^{-13}$ cm. sec.$^{-1}$ dynes^{-1}. The other fraction was chemically similar but was amorphous, and in the ultra-centrifuge was inhomogeneous, the principal components giving boundaries corresponding to $S_{w20^\circ} = 58 \times 10^{-13}$ and 220×10^{-13}. No explanation was put forward to explain these unusual results, but there seemed two that were most likely. One was that the heterogeneous end-product was a result of the method of purification, and the second that the virus culture was a mixture containing viruses with particles of different sizes, not all of which would crystallize. The work described in this paper was started to test these possibilities.

MATERIALS AND METHODS.

In most of the work two cultures of virus were used. One was originally supplied by Dr. Kenneth M. Smith in 1936, and had been maintained for four years at Rothamsted. The second was supplied by Dr. Smith from Cambridge in 1940, and was directly descended from that used by Pirie *et al.* (1938). These will be referred to as the Rothamsted and Cambridge cultures respectively. In addition, a few tests were made with virus found in naturally infected tobacco plants.

Unfortunately no plants are known whose leaves are infected systemically by tobacco necrosis virus, and the restriction of multiplication to local lesions

greatly hampers the production of virus in large quantities. The plants used were tobacco, var. White Burley, and French bean (*Phaseolus vulgaris*), var. Canadian Wonder. The same inoculum produces more lesions per unit area on bean than on tobacco leaves, but, in spite of this apparently greater susceptibility, the virus content of expressed sap from tobacco leaves is considerably higher than that from beans. Tobacco plants were therefore used for the production of virus and beans for the infectivity tests. When two preparations were being compared they were rubbed over opposite halves of the same

FIG. 1.—Leaf of French bean (*Phaseolus vulgaris*) infected with two serologically unrelated tobacco necrosis viruses. The left-hand half of the leaf was rubbed with the Rothamsted culture and the right-hand half with the Cambridge culture.

leaves, and when there were more than two each was rubbed over at least six half leaves chosen at random. Both bean and tobacco become increasingly resistant with age, and few lesions are obtained on old plants.

All the virus cultures used produced apparently identical symptoms Fig. 1), both on tobacco and bean, but when rubbed to opposite halves of the same leaves the Rothamsted culture produced rather more lesions than the Cambridge one on beans and less on tobacco.

Three antisera were used. One, that used by Pirie *et al.*, was supplied by Mr. E. T. C. Spooner. The others were prepared by Dr. A. Kleczkowski, one

against the Rothamsted and the other against the Cambridge culture. The rabbits were injected twice at five-day intervals with 1 mg. of purified virus, and were bled ten days after the second injection. All the antisera gave precipitin titres in excess of 1 in 500. The precipitin tests were made in tubes of 7 mm. diameter, which were kept in an illuminated water bath at 50° C. Usually 1 c.c. of serum at a constant dilution was added to a series of tubes, each containing 1 c.c. of antigen solution of different concentration, but some tests were made with constant antigen and varying serum.

The virus was purified in two ways. One was that described by Pirie *et al.* This readily gives a colourless product of high serological activity, but it involves the handling of large volumes of alcohol and the infectivity of the final product was found to vary widely. The following method gives an end-product whose chemical, physical and serological properties are apparently identical with those of the virus prepared by the other method, but its infectivity is more constant, and weight for weight is approximately as infective as virus in frozen, clarified sap.

Sap is expressed from necrotic tobacco leaves, frozen, thawed and centrifuged. Ammonium sulphate is added to the clear supernatant fluid at the rate of 250 g. per litre, and the resulting precipitate centrifuged off. This is resuspended in a volume of water equal to one-tenth of the original sap, brought to pH4 by the addition of acetic acid and centrifuged. The supernatant fluid is one-quarter saturated with ammonium sulphate, allowed to stand for an hour and then centrifuged. The precipitate is suspended in a volume of water equal to one-fiftieth of the original sap, and centrifuged to get rid of insoluble material. Saturated ammonium sulphate solution is now added to the supernatant fluid slowly until it becomes opalescent at room temperature, when it is placed at 0° C. After a few hours the preparation is centrifuged while cold, the precipitate is discarded and the supernatant fluid allowed to warm up to room temperature. The precipitate that now separates contains all the virus ; it is sedimented, resuspended in water, adjusted to pH6 with dilute NaOH, and any insoluble material is centrifuged off. The fractional precipitation with ammonium sulphate at 0° C. and room temperature is then repeated at pH6. The precipitate separating at room temperature is taken up in a small volume of water and dialysed. This sometimes produces a small precipitate, which is centrifuged off. The final preparation is colourless and slightly opalescent. 10^{-9} g. are usually sufficient to give lesions in beans, and 1 c.c. containing from $1/3 \times 10^5$ to $1/6 \times 10^5$ g. gives a precipitate visible to the naked eye when mixed with 1 c.c. of antiserum at 1/50.

SEROLOGICALLY UNRELATED VIRUSES AS THE CAUSE OF TOBACCO NECROSIS.

The first experiments were made with the Rothamsted virus culture. This was purified by the two methods to see whether the method of purification affected the homogeneity of the end-product. If the end-product separated into crystalline and amorphous fractions it was also intended to inoculate these separately to plants to test the possibility of their being separate strains, i. e. it was intended to see whether crystallization could be used as a method

of separating strains, and whether a wholly crystalline inoculum would give rise to a wholly crystalline virus preparation.

Both methods of purification gave the same end-product, one which would not crystallize, although many attempts to make it do so were made under the conditions described by Pirie *et al.* (1938). Samples were sent to Mr. N. W. Pirie, who also failed to obtain any crystals, and who found the preparation to behave like the amorphous fraction of his earlier preparations. This result seemed a fairly definite indication that there were different viruses or strains causing tobacco necrosis, some of which would crystallize and others not, and that the Rothamsted culture contained only the latter. The experiment was therefore repeated with the Cambridge virus culture. Again, however, the same result was obtained; regardless of the method of purification only an amorphous end-product was obtained. Both cultures have since been purified many times and no crystalline material has ever been isolated.

At first this seemed to make the proof of the existence of virus strains impossible, but when purified preparations of the Cambridge and Rothamsted cultures were tested against the three antisera differences were immediately detected between them, and positive evidence was obtained that the culture used by Pirie *et al.* was a mixture. The result of one such test is given in Table I. Preparations of both the Rothamsted and Cambridge cultures reacted with the serum used by Pirie *et al.* in 1938, but the Rothamsted culture failed to react with the serum made against the Cambridge culture in 1940

TABLE I.—*Precipitin Tests with Purified Preparations of the Rothamsted and Cambridge Virus Cultures and Three Antisera.*

A. *Rothamsted culture of tobacco necrosis virus as antigen.*

Antiserum.	Time.	Dilution of antigen. 1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.
Prepared in 1938 by Pirie *et al.*	10 mins.	+	++	+	−	−	−	−
	30 ,,	+++	+++	++	+	−	−	−
	180 ,,	++++	++++	++++	++++	+++	++	+
Against the Rothamsted culture	30 mins.	−	−	−	+	−	−	−
	180 ,,	−	++	++++	++++	+++	++	+
Against the Cambridge culture	24 hours	−	−	−	−	−	−	−

B. *Cambridge culture of tobacco necrosis virus as antigen.*

Prepared in 1938 by Pirie *et al.*	10 mins.	++	+	−	−	−	−	−
	180 ,,	++++	++++	++++	++++	+++	++	+
Against the Rothamsted culture	24 hours	−	−	−	−	−	−	−
Against the Cambridge culture	10 mins.	−	−	+	−	−	−	−
	180 ,,	++	++++	++++	++++	+++	++	+

+ signs indicate the degree of precipitation; − indicates that there was no precipitate. The dilution of antiserum was constant at 1/50 in 0·85 per cent. NaCl solution in all tests. 1/1 in the dilution of antigen equals 0·2 mg. of purified virus per c.c. Note that the position of the precipitation optima show that the antisera prepared against the Cambridge and Rothamsted virus cultures were richer in antibodies specific to them than the serum prepared against the mixed culture.

and the Cambridge culture failed to precipitate with the antiserum to the Rothamsted culture. Thus it was obvious that the disease tobacco necrosis can be caused by serologically unrelated viruses, and that the culture used by Pirie *et al.* contained at least two distinct viruses now separated into the Rothamsted and Cambridge cultures. As both of these failed to crystallize, it seems likely that the crystalline material represents yet another strain. As the two cultures used in this experiment originally came from the same source in 1936, the simplest explanation that can be offered for these results is that the culture then contained at least three viruses, one crystalline and the others amorphous, and that in continued sub-culturing the crystalline virus has been eliminated from both cultures, while one of the amorphous viruses has been eliminated at Rothamsted and the other at Cambridge.

Unfortunately it has not been possible to make any determinations of the sizes of the virus particles present in the two virus cultures, but the fact that the material purified by Pirie *et al.* gave a number of different sedimentation constants suggests that the serologically distinct viruses also have different particle sizes. Smith and MacClement (1940) found that tobacco necrosis virus behaved differently from any other studied in its filterability through collodion membranes. Although its filtration end-point was constant around 40 mμ., there was always a sharp drop in the infectivity of filtrates through membranes with pores of diameter between 125 mμ. and 250 mμ. This result is more simply explained by the theory that their virus culture was a mixed one, containing particles of various sizes, than on the theory of a reversible aggregation of particles of a uniform size, which they suggest. A culture of mixed viruses, in which the proportions of viruses with particles of different sizes was continually fluctuating, would also satisfactorily explain why the pore sizes of membranes causing this drop in infectivity varied with different batches of virus. Indeed it is probable that the change in filterability over a period of some months, noted by Smith and MacClement (1940), is an example of the unconscious selection of certain viruses from a mixture during continued culturing, such as that postulated above to explain the production of the Rothamsted and Cambridge cultures from a common stock.

Serological tests with cultures of virus causing tobacco necrosis found in naturally infected tobacco plants suggest that most of such cultures are mixtures and that the Rothamsted and Cambridge ones are also mixtures. Of five such infections tested, the virus from one reacted only with antiserum against the Rothamsted culture, but the other four reacted with serum against both cultures. The results of a test with one of these four are shown in Table II. This culture contains considerably more antigen capable of reacting with the antiserum to the Rothamsted than to the Cambridge culture. This is shown by the higher precipitation titre given in the constant antiserum test with the former serum, and by the large zone of inhibition by antibody excess in the constant antigen test with the antiserum to the Cambridge culture, which does not occur with the antiserum to the Rothamsted culture. In precipitation tests with mixtures of serologically related strains of other viruses such differences have never been noticed, for, although widely different precipitation optima may be obtained with different sera, the precipitation end-point in constant antisera tests is usually the same with homologous and heterologous

TABLE II.—*The Serological Reactions of a Virus Culture found in a Naturally Infected Tobacco Plant.*

A. *Constant antiserum test (serum dilution 1/40).*

Serum.	Time.	Antigen dilution. 1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.
Against the Rothamsted culture	1 hour	−	+++	++++	+++	++	+	−
	4 hours	+++	++++	++++	++++	+++	++	+
Against the Cambridge culture	1 hour	+	−	−	−	−	−	−
	24 hours	+++	++	+	−	−	−	−

B. *Constant antigen test (antigen dilution 0·05 mg. per c.c.).*

Serum.	Time.	Antiserum dilution. 1/10.	1/20.	1/40.	1/80.	1/160.
Against the Rothamsted culture	0·5 hours	+++	++	+	−	−
	24 ,,	++++	++++	++++	+++	++
Against the Cambridge culture	0·5 hours	−	−	−	−	−
	24 ,,	−	−	−	+	+

The sample of virus used was purified, and in the constant antigen test antigen dilution 1/1 equals 0·2 mg. of purified virus per c.c.

antisera. With other viruses precipitation titres have been found to be extremely valuable for quantitative work, but it is apparent from Table II that unless it is known that the serum contains antibodies to all the viruses present in the culture, the precipitation end-point of tobacco necrosis virus preparations may be little indication of the total amount of virus present.

Further evidence for the complexity of the virus cultures in naturally infected plants is the frequent occurrence of double zones in the precipitation tests. The proportions in which serologically distinct viruses occur in an infection may vary widely. Table III shows the effect of concentrating the virus from one naturally occurring infection. The clarified sap gave only one zone of precipitation and a titre of 1/8. After precipitating the virus

TABLE III.—*The Effect of Concentrating the Virus from a Natural Infection and the Production of Double Zones of Precipitation.*

Antigen.	Time.	Dilution of antigen. 1/1.	1/2.	1/4.	1/8.	1/16.
Clarified sap	30 mins.	−	+	−	−	−
	60 ,,	−	++	+	−	−
	120 ,,	+	+++	++	+	−

Antigen.	Time.	Dilution of antigen. 1/2·5.	1/5.	1/10.	1/20.	1/40.	1/80.	1/160.
Virus concentrated by $(NH_4)_2SO_4$	30 mins.	+	−	−	−	+	−	−
	60 ,,	++	+	−	+	++	+	−
	120 ,,	+++	+++	+	++	+++	++	+

The antiserum was that prepared against the Rothamsted virus culture and was used at a dilution of 1/50.

with ammonium sulphate and redissolving in a volume of water equal to one-twentieth of the sap, the preparation gave two zones of precipitation and a titre of 1/160. Here it is apparent that of the two strains present in the culture only one was present in the sap in sufficient quantity to give a reaction, whereas the other still reacted when the sap was diluted 1/8. Such double zones have never been seen in precipitation tests with other viruses which exist in strains, although some of these have been shown to be complex antigens, and their occurrence strongly suggests the presence of serologically distinct entities. It is possible that tobacco necrosis virus particles are themselves complex antigens, and this could explain some of the results with the naturally occurring infections. For example, if the Rothamsted culture contained only one type of virus particle these may have two antigenic groupings, A and B, and similarly the Cambridge culture might contain only one particle, but with two antigenic groupings, C and D. Then it is obvious that another culture containing a single virus with antigenic groupings B and C would react with antisera to both the Cambridge and Rothamsted cultures. However, as a virus particle must presumably precipitate as a whole and not as individual antigenic groupings, it is difficult on this theory to account for the widely different precipitin titres with different antisera (Table II). This possibility could only be tested by isolating numerous cultures of tobacco necrosis virus from single local lesions, in the hope that such cultures would contain only one type of virus particle, but even then the possibility of mutations that would alter the serological reactions would always arise. At present the results are most satisfactorily explained by regarding the viruses as simple and unrelated antigens.

THE INACTIVATION OF TOBACCO NECROSIS VIRUSES.

The type of precipitate given by all cultures of tobacco necrosis viruses has been the same and resembles that formed by tomato bushy stunt virus. The floccules are small and dense, and settle to form compact masses. Tobacco mosaic virus, on the other hand, forms loose nebulous floccules resembling those formed by bacterial flagella. Bawden and Pirie (1938) suggested that the difference between the precipitates of tobacco mosaic and bushy stunt viruses might result from the fact that the former has rod-shaped particles and the latter spherical ones. Work with further viruses has supported this suggestion, for of ten studied serologically, seven have been found to show anisotropy of flow, and so have anisodimensional particles, and all of these form the flagellar type of precipitate. Three do not show anisotropy of flow, those causing tomato bushy stunt, tobacco necrosis and tobacco ringspot, and so presumably have spherical or quasi-spherical particles, and these all give the same type of compact precipitate.

Tobacco necrosis also resembles bushy stunt virus (Bawden and Pirie, 1940) in that it is readily rendered non-infective without losing its serological activity. With viruses such as tobacco mosaic and potato " X " the separation of these two specific activities has been produced at all easily only by treatment with formalin, nitrous acid and hydrogen peroxide or by irradiation with X-rays or ultra-violet. With tobacco necrosis and bushy stunt viruses

the separation is produced by ageing, alkali and heating. Sap from plants suffering from tobacco necrosis loses its infectivity fairly rapidly at room temperature and is usually non-infective after 2–3 months, the exact time depending on the original virus content and the temperature. From such samples of old sap a non-infective protein can be isolated, which has physical and serological properties that are apparently identical with those of fully active virus preparations. Sap from healthy plants, of course, does not contain such a protein either before or after ageing. It is unknown what changes occur within the particles to cause them to lose their infectivity, and no tests have been made to determine whether bacteriologically sterile filtrates of infective sap or solutions of purifiéd virus would also lose their infectivity on standing at room temperature.

Purified preparations of both cultures of tobacco necrosis viruses lose their infectivity without otherwise altering their properties when treated with dilute alkali. The results of one experiment with the Rothamsted culture are given in Table IV, from which it will be seen that most of the infectivity was destroyed after 24 hours at 18° C. and pH 8, and all of it at pH 9, whereas at pH 10 there was no change in either the precipitation end-point or the optimal precipitation point. The non-infective preparations are fully active antigenically, and do not merely behave as haptens, for a rabbit injected with an alkali-inactivated preparation produced an antiserum that reacted in exactly the same manner as one prepared against a fully active preparation of the same virus.

TABLE IV.—*The Dissociation of Infectivity and Serological Activity by Alkali.*

pH.	Average number of lesions per half leaf.	Serological titre.
6	270	1/400,000
8	4·2	1/400,000
9	0	1/400,000
10	0	1/400,000

The virus samples used were from a purified preparation of the Rothamsted culture. Virus at 0·2 mg. per c.c. was exposed to the pH stated for 24 hours, then neutralized and tested. Infectivity tests were made at a virus content of 0·05 mg. per c.c., and the serological titre is the smallest weight of virus giving a visible precipitate.

The rate at which infective sap loses infectivity is greatly increased by heating, and sap that takes some weeks to become inactive at room temperature is inactivated by a few minutes at 70° C., some hours at 50° C., and days at 38° C. The effect of heating has been studied in greater detail with purified preparations, and it has been found that the loss of infectivity produced by heating at temperatures below about 85° C. has no effect on the serological reactions. Table V shows the results of heating crude infective sap and a purified preparation of the Rothamsted culture of tobacco necrosis virus at pH 6 for ten minutes at various temperatures ; the effects of heating a purified preparation of the Cambridge virus culture in pH 6 phosphate buffer for various times at 50° C. are also given. Only at 90° C. was there any fall in the serological titre, and at this temperature denatured protein separated from the

TABLE V.—*Effect of Heat on Tobacco Necrosis Viruses.*

A. *Heating the Rothamsted culture for ten minutes at various temperatures before and after purification.*

Temperature.	Crude sap. Average number of lesions per half leaf.	Purified preparation. Average number of lesions per half leaf.	Purified preparation. Serological titre.
Control	280	300	1/400,000
50° C.	109	216	1/400,000
60° C.	3	33	1/400,000
70° C.	1	1·5	1/400,000
80° C.	0	0	1/400,000
90° C.	0	0	1/400,000

B. *Heating a purified preparation of the Cambridge culture for various times at 50° C.*

Time of heating.	Average number of lesions per half leaf.	Serological titre.
Control	205	1/500,000
0·25 hours	111	1/500,000
1 hour	45	1/500,000
4 hours	20	1/500,000
16 ,,	1	1/500,000

All samples were heated in 0·1m. phosphate buffer at pH 6.

TABLE VI.—*Correlation between Loss of Infectivity and Serological Activity on Heating Potato Virus "X" for Ten Minutes at Various Temperatures.*

Temperature.	Serological titre.	Average number of lesions per half leaf at— 1/2.	1/20.
Control	1/256	230	108
59° C.	1/256	215	94
62° C.	1/128	130	29
65° C.	1/8	16	2·5
68° C.	No precipitate	0	0

The samples were heated in 0·1 m. phosphate buffer at pH 6. The infectivity tests were made on tobacco plants, the samples being tested at two dilutions. The serological titre is the greatest dilution at which 1 c.c. produced a visible precipitate when mixed with 1 c.c. of antiserum at 1/50.

preparation. The thermal inactivation point for ten minutes' heating was the same for both virus cultures. It was also the same for virus in crude sap and for purified preparations, but the infectivity of the former was reduced more by heating in the neighbourhood of 60° C., probably because of the separation of a precipitate of denatured plant proteins. It lies between 70° C. and 80° C., which agrees with the value given by Smith and Bald (1935), though it is considerably lower than that found by Price (1938).

The behaviour of tobacco necrosis on heating contrasts remarkably with that of potato virus "X." Table VI shows the results of heating this virus in

pH 6 phosphate buffer for ten minutes at various temperatures, and it will be seen that the loss of infectivity and of serological activity are parallel. Indications that a slight separation of infectivity from serological activity can occur with virus " X " were obtained by heating at pH 6 for long periods at 50° C., for after 16 hours the infectivity was reduced to one-quarter, whereas the precipitation end-point was only reduced to three-quarters of the control. This effect, however, is very small compared with tobacco necrosis virus (Table V), whose infectivity was reduced to 10 per cent. in four hours and to less than 0·05 per cent. of the control in 16 hours at 60° C., whereas the serological activity was unaffected. Differences in the behaviour of the two viruses are also clearly shown by the range of temperatures over which heating for ten minutes produces detectable effects. Tobacco necrosis, which is not completely inactive after ten minutes at 70° C., has its infectivity considerably reduced after ten minutes at 50° C. By contrast, potato virus " X " is not significantly affected by ten minutes at 59° C., but is completely inactivated by ten minutes at 68° C. It is apparent that the processes leading to loss of infectivity in the two viruses are different, for the inactivation of tobacco necrosis virus has a small temperature coefficient, and that of potato virus " X " a large one. Large temperature coefficients are characteristic of protein denaturation, and that this is closely linked with the inactivation of virus " X " is shown by the separation of a precipitate from the heated samples as they lose infectivity, and by the loss of anisotropy of flow. With all the plant viruses that have been studied, loss of serological properties has only been produced by treatments causing protein denaturation, whereas loss of infectivity can often occur as a result of slight intramolecular changes that have little effect on the gross physical and chemical properties. It seems that some such intramolecular changes occur in particles of tobacco necrosis when heated, treated with alkali, or aged in sap, whereas with potato virus " X " these treatments tend to cause the disruption of the particles.

DISCUSSION.

Serological methods have been of greatest value in plant virus studies in showing that viruses causing different symptoms may contain common antigens and so are related strains. The work described here shows that they can also be valuable in demonstrating that a disease, previously regarded as caused by a single entity, may be caused by a number of viruses. It has been known for some years that viruses with no common antigens can cause identical clinical symptoms in animals, for example foot-and-mouth disease, and this has been a complicating factor in their control, but no such diseases were known in plants. The demonstration that tobacco necrosis can be caused by a number of serologically unrelated viruses, therefore, is a further indication of the similarity between the viruses now too often separated into the two groups, plant and animal viruses. From what is already known it is obvious that the viruses placed in each group have widely different properties, and it seems probable that some of those attacking animals may have more in common with some plant viruses than with other animal viruses, and *vice versa*. The results described in this paper show that of two plant viruses, tobacco necrosis

and potato " X," one readily becomes non-infective while retaining its serological activity, but the other does not. It is likely that similar differences also exist between animal viruses, for such differences would explain why the heating or ageing of viruses produces successful vaccines with some, but not with others.

The serologically unrelated viruses causing tobacco necrosis used in this work all have similar stabilities and general physical properties, but it has already been suggested that they may differ in particle size, and it is possible that the disease can also be caused by viruses with other properties. For example, all the preparations described here gave amorphous precipitates in conditions similar to those in which Pirie *et al.* (1938) obtained crystals, and the virus used by Price (1938 ; 1940) and Price and Wyckoff (1939) appears to differ from any studied in England. This seems to be a homogeneous culture, for preparations sedimented in the ultracentrifuge with a single boundary corresponding to $S_{w20°} = 112 \times 10^{-13}$ cm. sec. $^{-1}$, dynes $^{-1}$, a value different from any found by Pirie *et al.* It also seems to be more resistant to heat and to alkali, for it was not completely inactivated after ten minutes at 90° C. or 24 hours at pH 9. It is noticeable, however, from Price's data that heating for ten minutes at temperatures considerably lower than the thermal inactivation point greatly reduced infectivity, and Price (1939) comments on the small temperature coefficient of thermal inactivation of tobacco necrosis virus compared with that of tobacco mosaic virus. The value of 4 for $Q_{10°}$, which he obtained, is almost certainly too small for the inactivation to have been a result of protein denaturation, but as he made no serological tests it is impossible to be sure that his culture behaved like those described in this paper.

It is difficult to decide what, if any, relationship these various viruses causing tobacco necrosis have with one another. In the past it has often been found that symptoms are quite unsuitable as a basis for identifying and classifying viruses, and instead their properties *in vitro* have been increasingly used. Serological reactions in particular have proved useful for demonstrating relationships, and it has become generally recognized that viruses reacting with each other's antisera are related strains and those that do not are unrelated. As these serologically unrelated viruses causing tobacco necrosis can differ in other than antigenic properties, it would seem preferable, for the present at least, to continue this practice and to regard them as separate viruses rather than related strains, although they cause identical diseases.

SUMMARY.

A method is described for the purification of tobacco necrosis viruses that leads to little or no loss of infectivity. Evidence is presented showing that tobacco necrosis is a disease that can be caused by a number of serologically unrelated viruses. All the viruses used failed to crystallize, and it is shown that the virus culture from which Pirie *et al.* (1938) obtained crystals was a mixed one. It is suggested that the serologically distinct tobacco necrosis viruses have particles of different sizes, and that they may differ in stability. The behaviour of the tobacco necrosis viruses on heating, ageing or treating

with dilute alkali differs from that of potato virus " X," for the former are rendered non-infective without being denatured, and they remain fully active antigenically.

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THE HEALING OF EXPERIMENTAL WOUNDS TREATED WITH EPICUTAN.

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ON the basis of Carrel and Baker's observations (1926) that embryo web porridge accelerated the healing of wounds, Fischer succeeded in producing an extract of calf embryo which is both sterile and stable and can be used as a dressing for wounds in man. This extract, absorbed on kaolin, was manufactured by Messrs. Lundbeck of Copenhagen under Fischer's direction, and marketed under the name of " Epicutan."

The effect of calf embryo extracts on experimental wounds in dogs (Nielsen, 1939) and of epicutan on accidental wounds in man (Waugh, 1940) has been investigated, and healing of the wound, as measured by the decrease in its surface area, is thought to be hastened. According to Fischer, epicutan also stimulates the growth of tissue cultures *in vitro*.

The present writers are not aware of any histological observations on the action of epicutan on wounds and, since Carrel's method of plotting the decrease in wound area is not wholly satisfactory, they have studied its effect on the various tissue processes involved in the healing of standard wounds in the rat (cf. Dann, Glücksmann and Tansley, 1941).

MATERIAL AND METHODS.

The effect of epicutan was studied on three types of standard wounds: superficial wounds, deep wounds and burns. The surgical and histological techniques were the same as those described in our previous paper (1941).

The fine epicutan powder was sprinkled on the surface of the wounds. Treated and untreated wounds were observed simultaneously on animals derived from the same mixed stock.

Superficial wounds.—First group : Fifteen animals were treated immediately after operation and then at 2-day intervals for 10 days. One rat was killed every day until the 8th day, and then at 2–3-day intervals until the 15th day. Two wounds became infected.

Second group : Six rats were treated for the first time on the 4th day, and five on the 8th day. These eleven animals were killed at 2–3-day intervals up to the 18th day, and at longer intervals until the 38th day. None were infected.

A further group of ten rats was treated with kaolin immediately after operation and then at 2-day intervals. None were infected.

Deep wounds.—Fifteen rats were used. The first application of epicutan was made immediately after the operation, a second on the 4th or 5th day, and a third about the 9th day. The animals were killed at 3–4-day intervals between the 4th and 50th days. Nine of the wounds were infected.

Burns.—Burns were made on 18 animals and were treated immediately after operation, and again about the 4th and 9th days. The rats were killed at 3–4-day intervals from the 3rd to 60th day. Four became infected.

The rate of infection was about equal for treated wounds (24·5 per cent.) and untreated wounds (23 per cent.).

RESULTS.

A. *Superficial wounds.*—Epicutan caused no marked improvement in the rate of healing as measured by the decrease in surface area during life (cf. Figs. 1 and 2). Histological measurements (see previous paper) fail to demonstrate any significant change in the rate of epithelialization of treated as compared with untreated wounds (Figs. 1 and 2).

The distance between the cut edges of the old collagen fibres shrinks to about 65 per cent. of the original defect within the first 24 hours. For the following four days, however, there is not such a marked contraction* in treated as in untreated wounds, but this difference has disappeared by the 8th day.

The regeneration of collagen fibres has a slightly longer latent period in the treated wounds, but when it has once begun it progresses better and more rapidly than in the untreated wounds. Epicutan not only accelerates, but improves regeneration of collagen fibres. The cavity of treated wounds was usually completely filled with new fibres by the 7th day, which never happened in the controls until the 10th day. The newly formed bundles in the deeper layers of the wound were considerably thicker than in the untreated wounds,

* It may be remembered that the decrease in distance between the margins of new epithelium and new collagen fibres represents a measure (although only in one dimension) of regenerative activity, while the decrease in distance between the edges of the old collagen fibres and old epithelium is a measure of the contraction of the wound, due only in later stages to regeneration (Dann, Glücksmann and Tansley, 1941). Since contraction causes the wound to assume a funnel shape, the curve of the decrease in distance between the cut edges of the epithelium does not run parallel throughout the period of healing with the corresponding curve relating to the collagen fibres.

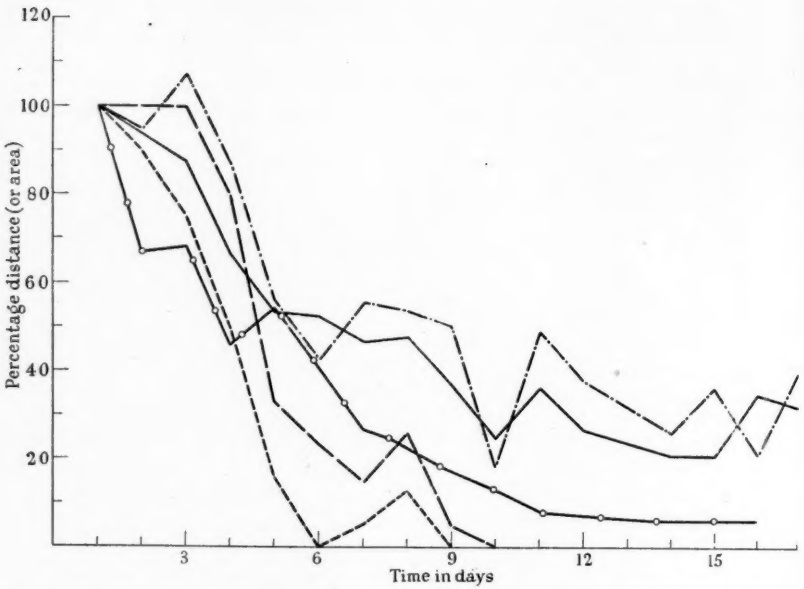

FIG. 1.—Measurements in superficial untreated wounds. ———— percentage distance between cut edges of old epithelium ; – – – – percentage distance between edges of regenerated epithelium ; —·—·— percentage distance between cut edges of old collagen bundles ; — — — percentage distance between edges of regenerated collagen bundles ; ——o—— percentage area measurements of wound surface *in vivo*. The average figure for the distance (or area) obtained 24 hours after operation is taken as 100 per cent.

while bundles nearer the surface were not affected. Microscopic measurements gave the following values for the average thickness of the largest regenerated bundles : (*a*) superficial layers—13·7μ in treated and 13·7μ in untreated wounds ; (*b*) deep layers—13·3μ in treated and 8·1μ in untreated wounds.

The elastic fibres were unaffected by epicutan.

Epicutan markedly improved muscle regeneration. Myocytes first appeared on the 6th or 7th day as in the controls, but muscle fibres differentiated as early as the 10th day, and thereafter were much more abundant and further developed than in the untreated wounds.

Infiltration by histiocytes and fibrocytes was the same in both treated and control wounds up to the 6th day, but later infiltration became much more abundant in the deeper parts of the treated wounds than in the superficial layers, while in the controls the condition was reversed. The increased average thickness of regenerated collagen fibres in the deeper layers of the treated wounds may be correlated with the increased fibrocytic infiltration of these parts.

When the first application of epicutan was made several days after the operation (Group II) instead of immediately (Group I), the reduction in the

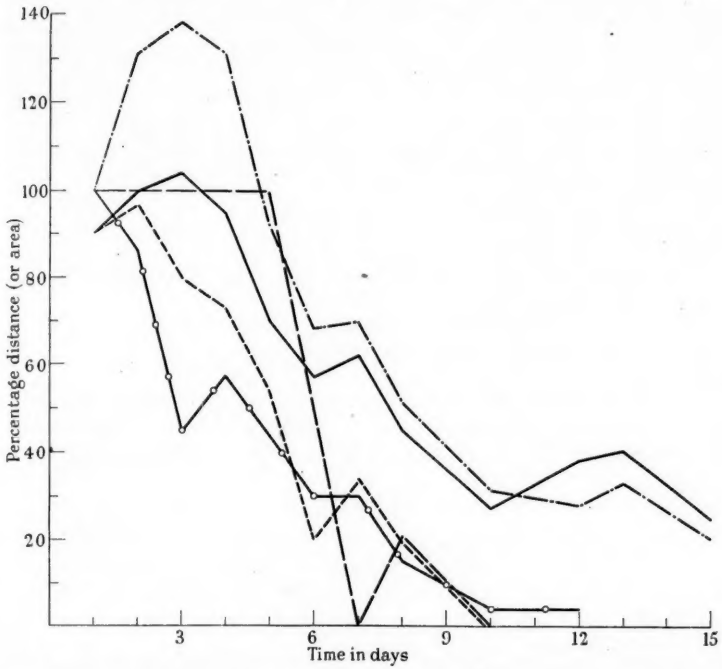

FIG. 2.—Measurements in superficial wounds treated with epicutan. ———— percentage distance between cut edges of old epithelium ; – – – – percentage distance between edges of regenerated epithelium ; —·—·— percentage distance between cut edges of old collagen bundles ; — — — percentage distance between edges of regenerated collagen bundles ; ——o—— percentage area measurements of wound surface *in vivo*. The average figure for the distance (or area) obtained in untreated superficial wounds 24 hours after operation is taken as 100 per cent.

distance between the cut edges of the old epithelium and old connective-tissue bundles proceeded at a slower rate (Table I).

TABLE I.—*Distances (as Percentages of Control Measurements made on the First Day) between Cut Edges of—*

	Old epithelium. Group.		Old connective-tissue bundles. Group.	
Days.	I.	II.	I.	II.
8	45	60	51	69
10	27	48	31	70
13	40	41	33	36
15	25	—	20	—
16	—	51	—	56

Muscle regeneration in Group II is, however, more active than in untreated wounds.

The rate of healing of superficial wounds treated with kaolin (which serves to absorb the calf embryo extract in epicutan) was the same as that of untreated wounds.

B. *Deep wounds.*—The results were essentially the same as for the superficial wounds. Skeletal muscle, like skin muscle, regenerated better and more rapidly in treated wounds.

C. *Burns.*—Epicutan produced little change in the external appearance of the burns.

In sections there was some indication that epicutan slightly stimulated epithelial growth, as the epithelial ring round the edge of the burn (see previous paper) was thicker and mitosis began earlier than in the controls. The wound surface was not covered any earlier, however, as a result of this increased epithelial growth.

As in superficial wounds, the regeneration of collagen was improved and muscle regeneration was active. New muscle fibres had differentiated in the treated burns by the 14th day as compared with the 24th day in the controls.

DISCUSSION.

In the present experiments epicutan did not produce any marked effect on the rate of decrease in wound area and rate of epithelialization. Whether Nielsen's most striking results are due to the fact that he experimented on dogs or whether his calf embryo extracts were in fact somewhat different from the commercial epicutan we are unable to say.*

Morosov and Striganova (1934) investigated the effect of human embryo extract on wounds in 62 rats. They claim positive results in 88 per cent. of their cases as regards decrease in wound area. Whether the extract was applied locally or by subcutaneous injection near the site of the wound was immaterial even though the control wounds were made on the same animal. They found that the histological effect was more marked in the regeneration of connective tissue than in that of epithelium, and they also claim improved vascularization of the treated wounds ; these histological findings resemble ours. Of the four graphs reproduced to illustrate the effect of their extract on decrease in wound size, two only show any significant change.

In the present experiments epicutan was found to have two main effects on the healing of wounds and burns, viz. to stimulate the formation of collagen and accelerate and improve muscle regeneration. As described elsewhere (Hughes, 1941), it also accelerates vascularization. Epithelial regeneration may be slightly affected, as evidenced by the fact that the epithelial ring around the necrotic tissue of burns was thicker in treated than in untreated burns.

Tissue culture experiments by E. N. Willmer, which will be reported in full elsewhere, have shown that *in vitro* epicutan stimulates the migration, but not the division of cells. This result may explain the fact, recorded above, that leucocytic and fibrocytic infiltration into the depths of the wound is

* Since this paper went to press, Young, Fisher and Young published a paper (*J. Path. Bact.* (1941), **52**, pp. 225–246) containing an admirable detailed criticism of the Carrell and Hartmann technique and of Nielsen's results based on this method.

increased with epicutan treatment. It may also account for the improved vascularization described by Hughes, since it is usually thought that capillary outgrowth depends on cell migration. The beneficial effect of epicutan on the regeneration of collagen and muscle may be secondary to the improved vascularization. In general our observations show that the effect of epicutan on experimental wounds in rats is quite beneficial. But in recent (unpublished) experiments better results have been obtained with cod-liver oil treatment, and much better results with the plaster-of-paris method except as regards muscle regeneration, for which epicutan is undoubtedly more effective. Since, however, muscle regenerates more readily in the lower mammals than in man, it would be unjustifiable to conclude from results obtained on rats that epicutan would also stimulate the regeneration of human muscle.

CONCLUSIONS.

1. In experimental wounds on rats epicutan does not greatly affect the rate of decrease in wound area nor the rate of epithelialization.
2. There is marked improvement of collagen and muscle regeneration in treated wounds.
3. Early application of epicutan proved more beneficial than its use at a later date.
4. The effect of epicutan treatment was inferior to that of certain other treatments in general clinical use.

We are very grateful indeed to Dr. H. B. Fell, Director of the Strangeways Research Laboratory, for her constant interest and advice during the investigation, and especially for her help in preparing the manuscript for press.

We are also indebted to Mr. V. C. Norfield, who made the graphs.

We wish to express our gratitude to the Royal Society for a grant to provide technical assistance, and to the Medical Research Council, by whom the general expenses of the investigation were defrayed.

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FIBROTIC NODULES IN THE TRACHEO-BRONCHIAL LYMPH GLANDS OF THE MOUSE PRODUCED BY BREATHING PRECIPITATED SILICA.

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In a recent research (Campbell, 1940) to study the effects of inhalation of silica dust upon the incidence of primary lung tumours in mice, the degree of dusting appears to have been such that the main effects of silica from the point of view of fibrotic nodule formation were in the tracheo-bronchial lymph glands whilst the lungs appeared to have escaped such nodulation. Since I can find no previous literature dealing with the inhalation of silica by mice or the effect upon their lymph glands, it seems worth while to record these effects in them and to report such fresh evidence, as has come to hand from them, upon various debatable points of silicosis as studied in other animals and in man.

METHODS.

Full details of the method of dusting are given elsewhere (Campbell, 1934, 1939). Seventy-five mice about three months old were exposed in a closed chamber to a moderate cloud of silica dust for about ten minutes once an hour for six hours on five days of each week for a year. The dust, as well as being inhaled, came into contact with the hair of the mice and was not brushed off except by the mice themselves. They inhaled the finer particles and clumps of particles, the majority of which were below 5μ in diameter; about 0·5 g. of silica per day entered each mouse-box containing 8 to 10 mice (Campbell, 1940). The mice were allowed to fulfil their allotted span of life, since the main object was the study of the incidence of primary lung tumours. Some of the mice died at various intervals before the year's dusting was completed. This enabled the effects of various lengths of exposure to silica to be observed. Microscopic sections were made of all lungs and tracheo-bronchial lymph glands and the sizes of the latter were also noted. Details of feeding and general care of the mice are given in the previous papers.

The silica used was the pure precipitated silica obtained from British Drug Houses. I am indebted to them for the following description of its preparation: The pure precipitated silica is made by adding nitric acid to a solution of sodium silicate and washing and drying the precipitate obtained. The solution of sodium silicate is well diluted and strong nitric acid is added; a jelly-like mass is obtained, and this is diluted with a further quantity of water and stirred until homogeneous; the mixture is adjusted to a definite acidity if necessary. The mass is then heated in a steam pan in order to granulate the

precipitate, which is eventually washed free from soluble salts. It is finally dried in air at a temperature of about 100° C. until the water content does not exceed 10 per cent. The product is then powdered to the required fineness and the final powder is produced in a high-speed mill. The silica does not appear to be amorphous, but consists of broken plates. The possible impurities, if any, are traces of soluble sodium salts not completely removed during the washing process. It is considered an advantage to use such a precipitated silica since it differs in some physical characteristics from crystalline quartz dust, which is the common cause of silicosis in man and which is used most commonly for experiments with animals. Positive results with such a precipitated silica tend to indicate that the effects of quartz dust are chemical rather than physical.

RESULTS.

A fairly early stage of the silicotic fibrotic nodule was observed in a mouse aged 264 days. As the mice were about 90 days old when the dusting commenced this mouse had inhaled the silica dust over a period of 174 days. Its tracheo-bronchial lymph glands were enormously enlarged, forming a mass measuring 15 mm. in width and 10 mm. in depth. This mass filled up a large part of the thoracic cavity, the enlargement being due to great increase in lymphocytes as well as to the presence of many—16 or more seen in one section—large collections, measuring up to 0·5 mm. in diameter, of silica cells or phagocytes containing silica dust. Most of these cells appear to be mononuclears, but there are a few multinucleated giant cells. Some of the cells appear to have died, lost their nuclei and become mummified. The reticulin is increased in quantity and has become thickened in places, where it appears to have changed to broader bands of hyaline fibrous tissue lying between the cells. There is no basket work or concentric arrangement of fibre at this stage. The enlargement of the lymph glands in this case was excessive, although 30 per cent. of the mice dusted showed some such hyperplasia (Campbell, 1940).

More advanced degrees of fibrosis were observed in mice aged about 600 days which had survived the whole year's dusting by about 100–150 days. Figs. 1 and 2 show two stages in the development of the fibrous tissue in the nodules in the same lymph gland of a mouse surviving the whole period of dusting by 123 days. Fig. 1 shows an earlier stage than Fig. 2. There are six nodules about 0·3 mm. in diameter and several smaller nodules in this same section. There is also some diffuse early fibrosis throughout the tissue. In all the larger nodules the main collections of dust cells are in the centre and the thickest fibres of hyaline substance are on the outside, where the dust is relatively scanty ; in short, where the fibres are thickest the silica seems most scarce. It certainly seems as if the fibrous tissue is attempting to confine the silica to the centre of the nodule. That such variations in degree of fibrosis exist in the different nodules of the same lymph gland seems to suggest that deposit of silica in different parts of the gland occurs at different periods in the dusting. The early stages of fibrosis in the lymph glands of the mouse resemble those described by Kettle (1934) for the lymph glands of the guinea-pig.

Multiple nodules with various degrees of moderate fibrosis are shown in Fig. 3 in a lymph gland from a mouse which survived the whole period of dusting by 132 days. Gardner (1932) published a similar illustration of multiple nodules in a rabbit. Fig. 4 demonstrates the formation of a basket-work of fibrous tissue in the lymph node of a mouse which lived for 138 days after cessation of the dusting. This section is stained with Robb-Smith's modification (1937) of the Foot and Mènard technique. A similar basket-work arrangement was obtained by Belt, Ferris and King (1940) in the rat, and by Gardner (1932) and Kettle (1934) in the guinea-pig.

More advanced fibrosis is found in the lymph glands of a mouse aged 736 days which survived the dusting period by 281 days. The nodules are about 0·5 mm. in diameter and show a fairly thick mass of silica cells and their remains in the centre supported by some fibrous tissue. Outside this central mass there is a well-developed capsule of coarse hyaline fibres (Fig. 5). Most of the fibres appear to form a lace-work, but in one part there is a tendency to arrangement in concentric layers. Silica is present only in isolated small quantities in this thick fibrous capsule so that the fibrous tissue seems redundant from the point of view of amount of silica present in its immediate vicinity. Fig. 6 shows a large area apparently clear of silica dust in the outer part of another nodule in the same lymph gland.

Belt, Ferris and King (1940) consider that such a redundant capsule of fibrous tissue is characteristic of the nodule in the human silicotic subject and they could not reproduce it in the rat. So far as I can judge, the only marked difference between the nodules in the mouse and those in man concerns their sizes. The largest nodule in the mouse in 0·5 mm. in diameter compared with the figures 1 to 5 mm. as the commonest sizes in the human subject.

About 50 per cent. of the 75 mice which inhaled the dust in the present experiment developed nodules in the lymph glands. The failure to produce nodules in all cases may be due to the presence of inhibitors against the toxic silica or the tissues may in certain cases develop some type of immunity. There is some confirmation for at least one inhibitor, namely, aluminium (Naeslund, 1940).

Another point of interest is that although the mice inhaled the dust and the dust had to pass through the lungs before reaching the tracheo-bronchial lymph glands, there was no evidence of fibrosis in the lung and yet well developed nodules were seen in the lymph glands. The degree of dusting was evidently not sufficient to affect the lungs also. Several observers have found that the earliest changes in silicosis tend to occur in the lymphatic apparatus (Gardner, 1932, 1940 ; Kettle, 1934 ; Simson and Strachan, 1940 ; Naeslund, 1940). A few other workers (Irwin, 1935 ; Belt, Irwin and King, 1936) report negative effects in lymph tissue.

DISCUSSION.

Crystalline or amorphous silica.

The experiments of Gye and Purdy (1922) and of Gye and Kettle (1922) indicate that crystalline silica is not essential for the production of fibrous

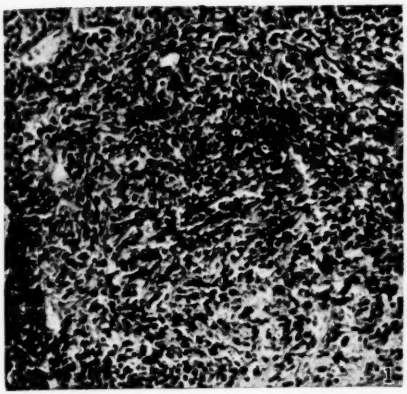
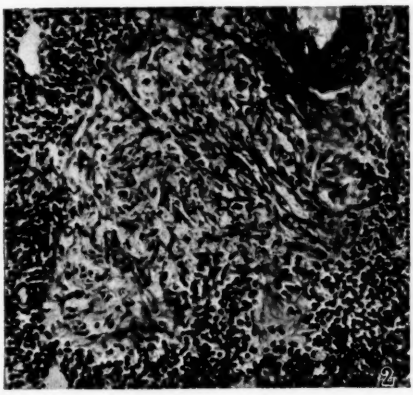
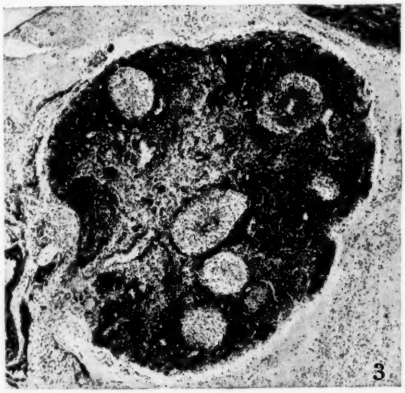
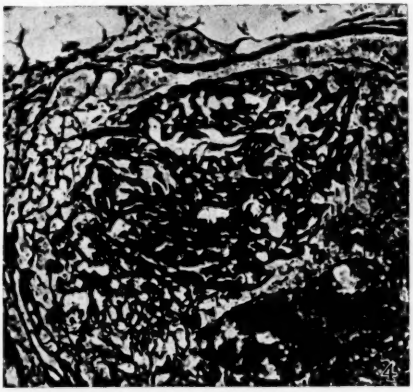
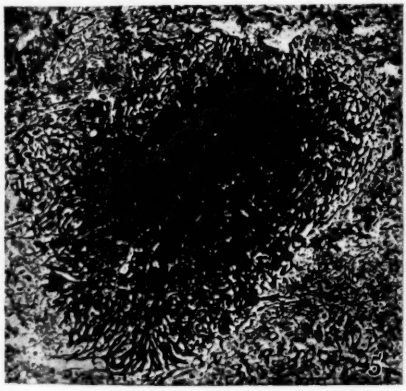
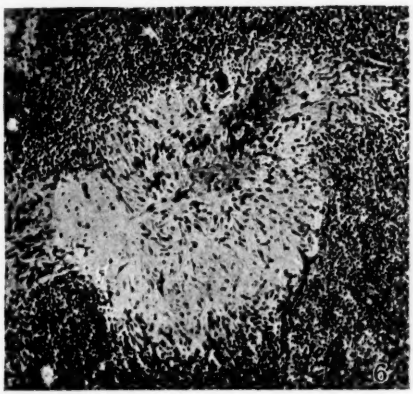

FIG. 1.—Early fibrosis in nodule. Weigert, Van Gieson. × 175.
FIG. 2.—Early capsule formation. Weigert, Van Gieson. × 175.
FIG. 3.—Multiple nodules. Weigert, Van Gieson. × 33.
FIG. 4.—Basket-work. Robb-Smith. × 130.
FIG. 5.—Redundant capsule. Robb-Smith. × 130.
FIG. 6.—Redundant capsule. Weigert, Van Gieson. × 85.

Campbell.

UNIV. OF MICH.

tissue and that colloidal silica is effective also. Gardner (1938) states that it is not the hardness of the particles that is essential, since diamond, the hardest known mineral, is not effective, nor is carborundum, which is also very hard. In the present work a precipitated silica has been used and it seems as effective as quartz. Drinker (1936) considers that there is no good reason to blame colloidal particles as the initiators of the silicotic nodule, since such nodules can be produced by particles approximating the common bacteria in size. However, it does seem possible that even in this case the silica is converted into a colloidal form before it becomes active.

Specificity and infective theories.

Briefly the specificity theory (Belt and King, 1938) holds that the fibrotic nodule is due to the effects of the silica in itself as the toxic agent. The infective theory holds that for development of the characteristic nodule, infection with tuberculosis is necessary in addition to the presence of silica. The mouse has been used in the present research and this animal, as is well known, is very resistant to tuberculosis, both human and bovine. Since with silica alone well marked fibrotic nodules have been obtained in the mouse's lymph glands, support is given to the specificity theory. Gye and Kettle's (1922) results from shorter experiments with colloidal silica upon the subcutaneous tissue of the mouse include a certain degree of fibrosis, although they found that when both silica and tubercle bacilli were present the lesions were more aggressive. Gardner (1940) reports that the toxic particles from silica, directly or indirectly through released phospholipids (Fallon, 1937), stimulate connective-tissue cells—fibroblasts, reticulum cells—in their immediate vicinity and proliferation ensues. Fallon explains the similarity between the silica and tuberculosis nodulations as being due in both cases to the formation of phospholipids in the cell, the silica poison and the tuberculosis toxin being separate primary irritants which cause liberation of phospholipids from the cells.

Miller and Sayers (1934) injected dust into the right side of the abdominal wall of the rabbit. The greater part of the dust was found in the peritoneum of the anterior abdominal wall. Of all the dusts they tested only those, e.g. quartz, chat and flint, containing large amounts of free silica produced a positive reaction with tumour masses of macrophages, fibroblasts and fibrous tissue cells.

Clark (1936) states that all animals tested—white rats, rabbits, guinea-pigs, monkeys, fowls, cats, tadpoles, fish—either by dusting or injection, show a tissue reaction to silica typified by the formation of fibrotic nodules except in the cold-blooded animals where there is necrosis followed by some fibrosis ; of all dusts tested only silica has a specific action upon animal tissue which results in fibrotic nodulation. To the above may now be added the present results from the mouse ; of several dusts, e.g. carbon (soot), iron oxide, and silica tested by inhalation, only silica has produced fibrotic nodules, although large quantities of the other dusts also reached the lymph glands (Campbell, 1939, 1940).

SUMMARY.

(1) By inhalation of a precipitated silica, fibrotic nodules were produced in the tracheo-bronchial lymph glands of about 50 per cent. of 75 dusted mice.

(2) The morphology of the nodules shows a resemblance to that for the classical fibrotic silica nodule in man.

(3) Fibrotic nodules were not produced in the lungs themselves, but this is considered to be due to the fact that most of the dust had been removed to the lymph glands and the degree of dusting was not sufficient to affect the lungs also.

(4) There is evidence that the silica is deposited in different parts of the gland at different periods.

(5) The results give support to the specificity theory for silicosis.

I am indebted to my colleague Dr. W. J. Purdy and to Mr. F. V. Welch of Mr. J. E. Barnard's department for assistance with the photographs; also to my technical assistant, Mr. C. Pergande, for help in this research, and to Mr. F. J. Higginson for cutting and staining the sections.

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OBSERVATIONS ON FACTORS CONCERNED IN SEDIMENTATION OF ERYTHROCYTES.

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CONSIDERATION of recent papers shows that the factors of prime importance in the rate of sedimentation of red cells are the concentration of protein in the plasma and the number of the red cells. It is also possible that the size of the red corpuscle and the lipoid content of the plasma may play a part, but the evidence on these points is less certain. It seems desirable, before reporting our findings, to make a short survey of what is known concerning the influence of the factors mentioned above.

The plasma proteins.

Fahraeus (1925) showed that in solutions of the plasma proteins prepared by precipitation with sulphates, sedimentation of red cells occurred with great rapidity in pure fibrinogen, less rapidly in globulin and extremely slowly in albumin. Further evidence of the influence of fibrinogen and globulin in causing the erythrocytes to sediment rapidly is afforded by observations that after defibrination of the blood a rapid erythrocyte sedimentation rate (E.S.R.) is reduced to within normal limits, unless the plasma globulin is also above normal. The influence of the respective protein fractions of the plasma on the E.S.R. has been confirmed in experimental studies by Zarday and von Farkas (1931) and by Kylin (1935). Recently Tiffeneau and Gysin (1937) have shown that in a protein-free dialysate of plasma, sedimentation is almost *nil*. Furthermore, it has been shown by Zarday and von Farkas (1931) and Coburn and Kapp (1936) that addition of fibrinogen or globulin to plasma increases the E.S.R. of cells suspended therein, while albumin has the opposite effect.

The relation between the plasma protein fractions and the E.S.R., so clearly demonstrated in the experimental work just described, is less obvious and less widely accepted in studies made on patients. It is well recognized that plasma fibrinogen and also globulin are increased by infectious processes and when tissue destruction is occurring, i. e. in just those conditions in which the E.S.R. is above normal ; yet nevertheless many workers have been unable to detect a close relationship between a rise in E.S.R. and increase in globulin and fibrinogen of the plasma. Pinner, Knowlton and Kelly (1928), for example,

* Work done during the tenure of a Hall Fellowship.
† Work done during the tenure of a Carnegie Teaching Fellowship.

found that there was little parallelism between the plasma fibrinogen and the E.S.R. in pulmonary tuberculosis, apart from the fact that the E.S.R. was never very rapid when fibrinogen was within normal limits. In acute infectious diseases Oelkers and Ohnesorge (1929) found no relation between globulin and E.S.R. Nitschke (1931) in various diseases and Aldred-Brown and Munro (1935) in chronic rheumatism found that the changes in the plasma proteins were not related in any way to changes in the E.S.R. The figures of Hartung, Davis, Steinbocker and Straub (1936), and of Davis (1935), for chronic rheumatism, of Moen and Reimann (1932) for pneumonia, of Jones (1929) in various conditions, all show a tendency for fibrinogen and globulin to be high when the E.S.R. is more rapid than normal; but in none of these series were the figures subjected to statistical analysis, and in all the number of observations is relatively small.

On the other side of the scale is the evidence provided by those employing statistical analysis of their data. Westergren, Theorell and Widestrom (1931) analysed in this way the results of 100 observations on 84 cases and found that fibrinogen and, to a lesser extent, globulin showed a significant positive correlation with the E.S.R. while albumin showed a slight negative correlation. In other words a rise in fibrinogen and globulin and a fall in albumin were associated with rates above normal. Bendien and Snapper (1931) also found a comparable statistical relationship between fibrinogen and globulin and the E.S.R. in a large series. Contrary to the results of Westergren *et al.* (1931) they found that the percentage of albumin had no influence on the E.S.R. The results of Greisheimer, Johnston and Ryan (1929), Gilligan and Ernstein (1934), and Ham and Curtis (1938) also indicate a significant positive correlation between fibrinogen and the E.S.R. More recently Lucia, Blumberg, Brown and Gospe (1936) found that when serum albumin was low the E.S.R. was high and *vice versa*.

The evidence may be summed up as follows: When statistical studies have been made on a large series of cases a significant correlation can be determined between the E.S.R. and plasma fibrinogen and globulin, high rates being associated with increased values of these substances. There is also some evidence that a fall in plasma albumin is associated with an increase of the E.S.R.

The red cell factor.

Fahraeus (1925) showed that the erythrocytes played a secondary part to the plasma proteins by the following experiment: He suspended red cells from a patient with a rapid sedimentation rate in plasma from an individual with a normal E.S.R. and *vice versa*. In the former experiment the E.S.R. was normal, but in the latter rapid, clearly showing that the red cells *per se* had no influence on the sedimentation rate. Fahraeus (1925) also showed that a reduction in the red cell concentration resulted in an increase of the E.S.R., a finding which has been confirmed experimentally by many workers. In patients with anaemia Bendien and Snapper (1931) and others have found a similar state of affairs, and many authorities hold that in all cases with an abnormal red cell count a correction must be made in order to obtain a true

idea of the E.S.R. This correction, as Ham and Curtis (1938) state, is not universally practised. According to them the Westergren (1920) procedure is seldom "corrected"; Bannick, Gregg and Guernsey (1937), who employ it, believe that the effect of anaemia is less marked than that produced by an equivalent dilution *in vitro*, and accordingly over-correction is a frequent result of attempts to allow for anaemia. Bouton (1938) found that the effect of anaemia after haemorrhage was slight and inconstant; correction was fallacious, and in fact the E.S.R. and the red cell count frequently moved in the same direction. In the opinion of the majority of workers, however, there seems to be no doubt that a fall in the red cell concentration is associated with a rise in the E.S.R. In slight degrees of anaemia the effect on the E.S.R. is small, and it is probable that at any rate with the Westergren technique correction is not sufficiently accurate to make it worth while.

Confirmation that the size of the erythrocytes has some bearing on the E.S.R. has been afforded by the experimental work of Ham and Curtis (1938), who found that microcytes suspended in normal plasma sank more rapidly than normocytes, but less rapidly than macrocytes. In this experiment the total volume of red cells in each case was the same. According to Ham and Curtis (1938) no correction can be made for this abnormality.

The lipoid factor.

In vitro a rapid E.S.R. in association with a high plasma cholesterol has been reported, while a decrease in the rate has been found with a high lecithin value. Theorell (1930) was unable to confirm these findings in patients, however, and Ohlson and Rundquist (1932) found the E.S.R. in plasma made "lipoid-free" to be the same as in the untreated plasma containing much lipoid. In defibrinated blood, with normal plasma globulin, Ham and Curtis (1938) found a high cholesterol in association with a rapid E.S.R. in eight cases of nephrosis and diabetes. There was, however, no correlation between the level of the cholesterol and the E.S.R. From the slight evidence available it cannot be concluded that there is any proof that an elevation of plasma lipoids is in any way responsible for changes in the E.S.R.

OWN OBSERVATIONS.

In this paper are reported 220 observations, obtained from 58 individuals, on the erythrocyte sedimentation rate, the red cell volume, and on the fibrinogen, globulin and albumin of the plasma. Using the first observations from each of the 58 individuals of the series, Dr. P. L. McKinlay, of the Department of Health for Scotland, has calculated the inter-relationships of the various factors assumed to be involved in the sedimentation of erythrocytes.

The data were obtained from the following:

(1) 51 observations on 12 patients suffering from rheumatoid arthritis.

(2) 57 observations on 8 patients obtained before and after operation for hernia.

(3) 99 observations on 34 patients, in the form of curves made fasting, 40 minutes and 3 hours after ingestion of protein, glucose or fat. In 3 instances no sample was obtained at 40 minutes.

(4) 13 observations made on 4 patients who received injections of T.A.B. vaccine.

Methods.

The technique used in the estimation of the plasma proteins was that of Howe (1921), as modified by Hawk and Bergeim (1938). Non-protein nitrogen was estimated by the method of Folin and Wu (1919) and the E.S.R. by the procedure of Westergren (1920). Red cell volume was estimated by using graduated centrifuge tubes. The anti-coagulant was potassium oxalate, one drop of 15 per cent. solution to 5 c.c. of blood.

Normal controls.

The values for 14 "normal" controls, members of the staff for the most part, are given in Table I. These figures agree with those of previous workers. Readings for the E.S.R. were not obtained on these subjects. Westergren (1920), whose technique was employed, gives 3 mm. per hour as the upper limit of normal.

TABLE I.—*Plasma Protein Values and Red Cell Volume in 14 Normal Controls.*

	Protein—g. per 100 c.c.				Red cell volume (per cent.)
	Total.	Albumin.	Globulin.	Fibrinogen.	
Maximum	7·91	4·84	3·16	0·46	48·3
Minimum	6·18	3·99	1·78	0·14	39·3
Average	7·19	4·54	2·38	0·27	44·7

Results.

Arthritis patients.

In the twelve patients in this group suffering from rheumatoid arthritis the E.S.R. was high throughout the period of observation: fibrinogen usually, and globulin often, were above normal. The most consistent abnormality, however, was a drop in the plasma albumin. As other workers have noted, inspection of the figures did not suggest any very close correlation between the E.S.R. and the plasma protein fractions.

Surgical patients.

It has been shown that even the slight degree of damage to tissue produced by herniotomy is followed by a rise in the E.S.R., while Govaerts and de Harven (1928) found that a rise in plasma fibrinogen always occurs. Similarly Cuthbertson and Tompsett (1935) noted a rise in globulin and a fall in albumin after manipulation of fractures. In view of these facts we investigated changes in the plasma proteins and the E.S.R. in eight patients before and after operation for herniotomy in order to detect the presence of any relationship between them. Fifty-seven observations were made on the eight patients. Table II shows a typical case. Four to ten days after operation the E.S.R. reached its maximum, which ranged from 8 to 23 mm. in one hour. At least a fortnight

elapsed before the E.S.R. returned to normal. The changes noted in the plasma protein were as follows: fibrinogen rose above normal while globulin also tended to increase; albumin usually fell slightly.

TABLE II.—*Effect of Double Herniotomy on the Plasma Proteins and E.S.R.*

Date.	g. per 100 c.c.					Erythrocyte sedimentation rate (mm./1 hr.).	Red cell volume (per cent.).
	Total protein.	Albumin.	Globulin.	Fibrinogen.	Non-protein nitrogen.		
3.x.40	7·81	4·97	2·61	0·23	33·3	3	46·6
4.x.40*	7·23	5·30	1·75	0·12	34·1	3	45·3
4.x.40†	7·60	4·02	3·49	0·09	44·1	2	46·9
5.x.40	6·28	3·90	2·4	0·24	53·8	2·5	45·5
6.x.40	6·92	3·72	2·37	0·83	31·7	5·0	46·3
7.x.40	6·57	3·67	2·19	0·71	29·0	12·0	43·0
8.x.40	6·75	3·73	2·41	0·61	30·2	20·0	42·9
10.x.40	6·93	4·08	1·58	1·27	24·0	23·0	40·9
12.x.40	6·13	3·65	1·89	0·61	27·1	21·0	40·5
14.x.40	5·94	4·08	1·30	0·56	26·4	16·0	42·3
17.x.40	7·53	4·10	2·03	1·30	24·6	11·0	41·6
21.x.40	7·86	4·28	2·83	0·75	21·7	6·0	41·3
25.x.40	7·63	4·31	3·09	0·38	27·6	9·0	40·8

* Before operation.
† 4 hours after operation.

Seven other patients showed similar changes, which, though not great as regards the E.S.R., are definite. There would appear to be some correlation between the fibrinogen and the E.S.R. in this group.

Ingestion of food.

It has been reported that ordinary meals are without influence on the E.S.R. (Rourke and Plass, 1929). On the other hand, the lipoid content of the plasma has been reported as influencing the E.S.R., and we decided to try the effect of ingestion of sufficient fat to render the plasma definitely lactescent. No estimation of the blood lipoids was made. 180 c.c. of 40 per cent. cream were given to eight fasting subjects and observations made on the E.S.R. and the plasma protein fractions, fasting, 40 minutes and 3 hours after ingestion. At 3 hours there was marked lactescence of the plasma in every instance. No change occurred in the E.S.R. whether the fasting level was normal or abnormal. As regards the plasma proteins, a slight rise in albumin usually occurred, fibrinogen remained unchanged, while globulin was inconstant. Olive oil, 120 c.c., was given to five fasting patients. The E.S.R. here also was unaffected and no constant variation occurred in any of the plasma protein fractions. From these results it seems that lipaemia, at least that of a fleeting nature following ingestion of fat or olive oil, has no effect on the E.S.R.

Although the rather meagre evidence is against the view that food affects the E.S.R., and Cherry (1934) found that a meal of 150 g. of lean meat produced no change, none the less it has been suggested that fibrinogen may be temporarily increased in the human subject by the ingestion of protein, as Foster and Whipple (1921) found to be the case in the dog, although it is to be noted that relatively very large amounts of protein were given in the experiments over a period of days. We therefore decided to see if any change

occurred in the plasma proteins, and therefore possibly in the E.S.R. following ingestion of a large protein meal. For this purpose 40 g. of protein—120 g. of chicken and 2 eggs—were given to 15 fasting subjects. Observations, fasting, 40 minutes and 3 hours after ingestion gave no evidence of change in plasma fibrinogen or the E.S.R., whether the fasting levels were normal or abnormal. A tendency to a slight fall in albumin and rise in globulin was noted.

Cherry (1934) noted that the E.S.R. tended to vary inversely with the blood sugar after ingestion of 1·7 g. of glucose per kilo body weight. To complete the experiment we next gave 100 g. of glucose in 100 c.c. of water to six subjects. No significant variations were noted in the E.S.R. or in any of the protein fractions.

There is thus no evidence that protein, fat or glucose in the amounts given by us influence either the normal or the abnormal E.S.R.

The effect of protein shock.

The remaining 13 observations which comprise part of the 220 in this analysis were made on four patients who received protein shock treatment by injection of T.A.B. vaccine intravenously. This is said to be followed by a rise in serum globulin (Lloyd and Paul, 1929). The observations were made as a rule at 4 hours (when fever was usually at its height) and 24 hours after injection. Single injections of vaccine produced no change in the E.S.R. nor in the protein fractions. After repeated injections in one case the red cell volume during that period fell from 39·6 to 32·3 per cent., indicating anaemia as the probable cause of the rise in E.S.R. which had occurred.

The red cell volume.

Haematocrit readings were made in all the patients. In patients with arthritis the level was below 39·3 per cent. in most instances, the anaemia being of the microcytic type, as indicated by Pijper's halometer. In the surgical group the operative procedure was not followed by any gross change in the red cell volume as blood loss was slight. Ingestion of fat, protein or glucose had no effect on the red cell volume. Repeated protein shock, in one of the four cases, was associated with a definite fall in the red cell volume.

ANALYSIS OF 220 OBSERVATIONS ON THE PLASMA PROTEIN FRACTIONS, THE ERYTHROCYTE SEDIMENTATION RATE (E.S.R.) AND THE RED CELL VOLUME.

In Figs. 1–5 have been plotted the plasma protein fractions—albumin, globulin, fibrinogen and total protein, and the red cell volume against the E.S.R. The following method of depicting the results is adopted. The protein fractions are arranged in groups and the average for each group found; the corresponding E.S.R. values in these groups are also averaged. The number of observations at the ends of the curves are in many instances few and therefore the figures have been "smoothed."

Fig. 1 shows an obvious inverse relationship between plasma albumin and

the E.S.R. Fig. 2 shows a moderate degree of direct relation between globulin and E.S.R. Fibrinogen and E.S.R. also show a direct relationship (Fig. 3).

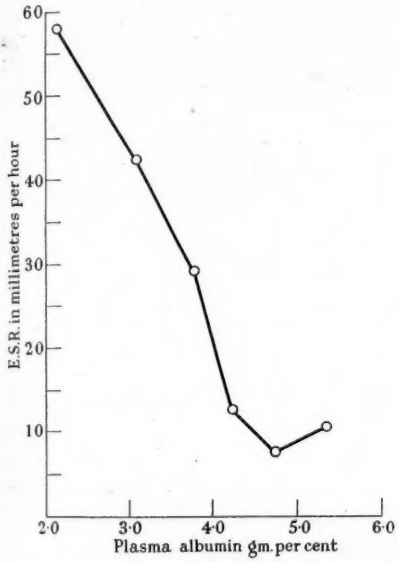

FIG. 1.—Relation between plasma albumin and erythrocyte sedimentation rate.

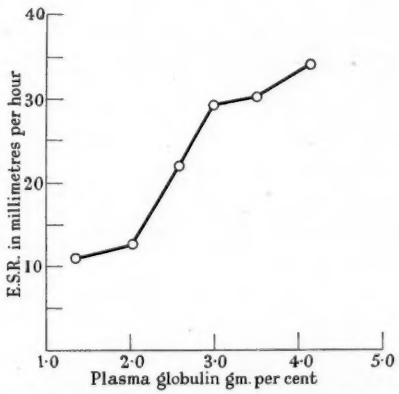

FIG. 2.—Relation between plasma globulin and erythrocyte sedimentation rate.

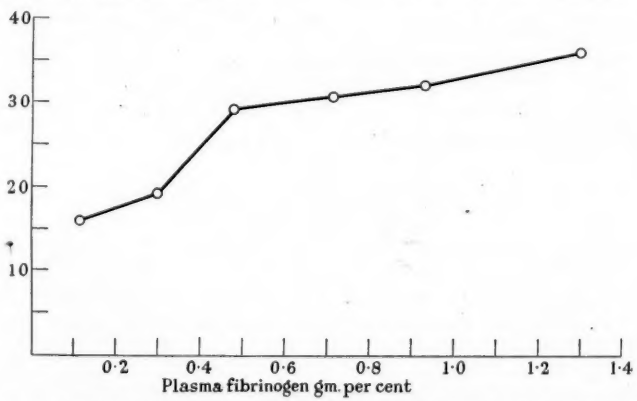

FIG. 3.—Relation between plasma fibrinogen and erythrocyte sedimentation rate. Ordinate = E.S.R. in mm. per hour.

There is, however, no apparent relation between total protein and the E.S.R. (Fig. 4). In Fig. 5 a close inverse relationship is seen between the red cell volume and the E.S.R.

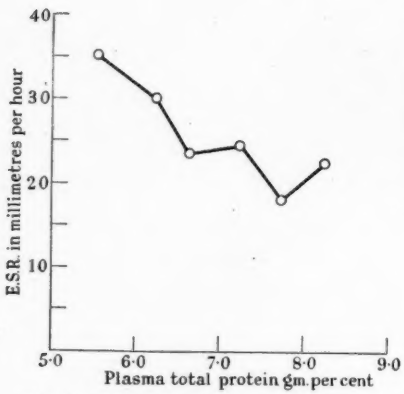

FIG. 4.—Relation of plasma total protein to erythrocyte sedimentation rate.

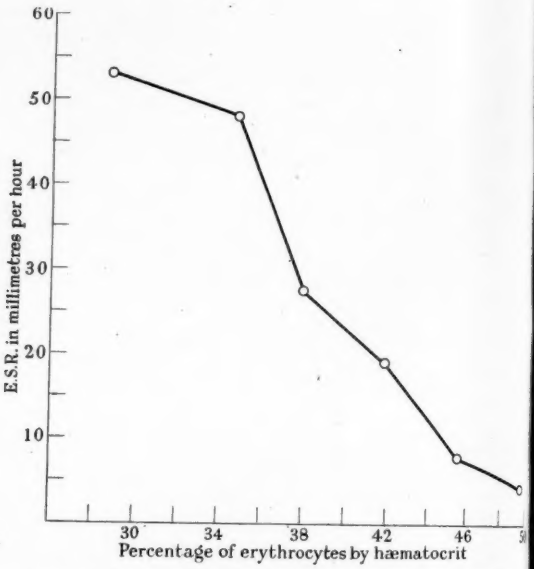

FIG. 5.—Relation between red cell volume and erythrocyte sedimentation rate.

*Statistical Analysis.**

Owing to the heterogeneous nature of the data it was thought desirable, for statistical purposes, to use only one observation from each individual. For the fifty-eight individuals examined the first determination of each variable has been used to calculate the inter-relationships of the several factors. The coefficients of correlation are given in Table III.

TABLE III.—*Coefficients of Correlation.*

	Red cell volume.	Albumin.	Globulin.	Fibrinogen.
Erythrocyte sedimentation rate	—·581	—·693	·376	·365
Red cell volume	..	·573	—·273	—·276
Albumin	..	..	—·527	—·230
Globulin	..	..	..	—·116

It will be observed that the sedimentation rate is significantly correlated with each of the other factors under review, inversely in the case of albumin content and red cell volume and directly with globulin and fibrinogen contents, the coefficients in these two latter instances being rather lower than those

* For this we are greatly indebted to Dr. P. L. McKinlay of the Department of Health for Scotland.

for red cell volume and albumin content. The higher the red cell volume and albumin content of the plasma the lower on average is the E.S.R., and the higher the globulin and fibrinogen contents the higher on average is the E.S.R.

Inspection of the other coefficients of correlation indicates, however, that the four variables—red cell volume, albumin, globulin and fibrinogen—do not vary independently, particularly so in the case of red cell volume and albumin, which tend to vary directly with one another, and in respect of albumin and globulin, which tend to vary inversely with one another; the higher the albumin the lower generally the globulin content of the plasma. To determine the relationship between the E.S.R. and any one of the remaining variables for constant values of the others, coefficients of partial correlation have been calculated, these being shown in Table IV:

TABLE IV.—*Coefficients of Partial Correlation.*

Variability.	Constant.	Coefficient of partial correlation.
E.S.R. and red cell volume .	Albumin, globulin, fibrinogen .	—·275
,, ,, albumin . .	Red cell volume, globulin, fibrinogen .	—·444
,, ,, globulin . .	,, albumin, fibrinogen .	·114
,, ,, fibrinogen . .	,, ,, globulin .	·274

After elimination of any indirect influence of other variables it will be noted that—

1. Fall in albumin is associated with a rise in E.S.R.
2. To a lesser extent fall in red cell volume is associated with a rise in E.S.R.
3. Rise in fibrinogen is associated with a rise in E.S.R.
4. Rise in globulin has but only slight influence.

This analysis confirms the view that the erythrocyte sedimentation rate is influenced by the red cell volume and by certain protein fractions of the plasma.

SUMMARY.

The erythrocyte sedimentation rate (E.S.R.) was uninfluenced by ingestion of fat in one meal sufficient to render the serum lactescent. It was also unaffected by ingestion of 40 g. of protein, or 100 g. of glucose.

After the operation of herniotomy, in each instance a rise in the E.S.R. was accompanied by a fall in albumin and an increase in the fibrinogen and globulin content of the plasma.

From analysis of 220 observations on 58 individuals it appeared that the E.S.R. becomes more rapid with a fall in the albumin content of the plasma, with anaemia as measured by the volume of packed red cells and with increase of the fibrinogen and of the globulin content of the plasma. Statistical analysis confirmed these findings. Considered as single factors, fall in albumin was most closely associated with increase in E.S.R. This increase in rate was

to a lesser degree associated with fall in red cell volume and with increase of plasma fibrinogen. Between the globulin content and the E.S.R. there was no appreciable relationship.

We wish to thank Dr. George Allan, Dr. John Gracie and Mr. Roy F. Young for the opportunity of studying some of their patients.

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SPREAD OF INFECTION FROM THE RESPIRATORY TRACT OF THE FERRET. I. TRANSMISSION OF INFLUENZA A VIRUS.

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THERE is general recognition of the fact that infective agents may pass from the respiratory tract of one host to that of another through the air. There has, however, been but little study of the precise mechanism of this transmission under experimental conditions. The term "droplet infection" is in common use; this includes infection caused by the scattering of fairly large drops from the mouth or nose. Wells and Wells (1936) have lately emphasized the importance of droplet nuclei: droplets which are fairly large as they leave the mouth are expelled with such speed that they rapidly lose fluid by evaporation until they become so small that they can float in the air for many hours. Wells and Wells have urged that for this reason "droplet nuclei" form a greater source of danger than the coarse particles. Infection may be carried in the air in yet a third way, on particles of dust. The relative importance of the various potential vehicles of infection is hard to determine under field conditions. We have therefore attempted to study the problem experimentally, deliberately avoiding the somewhat artificial conditions produced by spraying suspensions of organisms into the air. We began by investigating aerial transmission of influenza A virus in the ferret, as we know of no other infection more suitable for our purpose.

Experimental Methods.

Ferrets under investigation were housed in the experimental animal isolation hospital at Mill Hill. This contains 32 separate cubicles 6 ft. 9 in. by 5 ft. 9 in. by 9 ft. by 9 in. high, each provided with a window facing the door. Each experiment was carried out in a separate cubicle, and the walls and floors of each cubicle were washed down with lysol followed by water from a high-pressure hose between two experiments. For the experiments on direct contact ferrets were housed in special cages—metal-sided boxes 1 ft. 6 in. by 1 ft. 6 in. by 1 ft. 6 in. high, with perforated metal lids; for other trials open wire-meshed rabbit cages were used. Attendants wore rubber boots and mackintoshes which were kept wet with lysol; care was taken by dipping hands, tongs, thermometers and so forth in lysol to prevent mechanical transfer of infection from one animal to another.

Infection by Immediate Contact.

It is known from earlier work (Stuart Harris, Andrewes and Smith, 1938) that ferrets running together on the floor of a cubicle will transmit infection from one to another. We readily confirmed that close contact regularly permitted cross-infection, and also paid attention to the possibility that repeated transfer in this manner might modify the properties of the virus. A normal ferret was placed in a metal cage with one which had been infected 24 hours previously with a ferret-adapted strain of influenza A virus (WS). Four days later the originally infected ferret was removed and another clean one introduced. The second ferret duly sickened with influenza and proved capable of passing the infection to the third. In a similar manner the contact series was carried on through eight ferrets, the period of contact of normal and infected ferrets varying from two to four days. All ferrets developed fever of 104·6° F. or higher with typical nasal symptoms: no suggestion of modification of the virus was forthcoming.

When a normal and an infected ferret have been placed in adjacent metal cages infection has usually failed to pass from one to the other. We have not, however, relied in practice upon the negative results of the few experiments which were designed to test this point; experimental ferrets are kept, except for special reasons, in cages each in a separate cubicle.

Infection by Exposure at a Distance.

Experiments were next carried out to determine over what distance a ferret with influenza could project infected particles in sufficient numbers to convey the disease. Ferrets were placed in the isolation cubicles already mentioned and were housed in open wire-mesh rabbit cages on racks. In earlier experiments one infected or "donor" ferret was placed in each cubicle; later we used two "donors" and two normals or "recipients." The two donors were kept in one cage—a proceeding which apparently increased their activity. In order to decrease the chance of accidental transference of infection by human agency, temperatures of the donors only were taken, except in the earliest trials, until a typical febrile response occurred in them; thereafter recordings on the recipients were begun and confined to these. Table I shows the results of eleven experiments. A decision as to whether any given ferret had "taken" or not was based on the temperature response, the symptoms and the result of a subsequent immunity test to the same (WS) strain of virus. The last was particularly necessary, as many exposed ferrets developed a milder disease than that following intranasal inoculation of virus. It will be seen that, in three experiments, only one of two recipients contracted the disease; this indicates that in such experiments the amount of virus passing across the cubicle must have been near the threshold dose. Further, in experiments 2 and 4, one recipient became infected three days after the other and may have caught the disease from his neighbour rather than from the donors. Very probably cross-infection from one recipient to the other

would have occurred also in experiments 3, 8 and 10 if their contact had been longer continued.

Table I shows that with one donor ferret in a cubicle, infection of one or both recipients occurred in two out of three trials; with two donors present, six out of eight experiments were positive. Transmission took place in seven tests over an air-distance of 5 ft.; longer distances could not be tested conveniently in the cubicles available. In order to diminish the chances of effective projection of coarse droplets by a sneezing ferret, recipients were in most trials placed at a higher level than donors; there is evidence that the larger particles settle before they have travelled more than 1 or 2 ft., though we cannot say that the infection of a normal ferret placed 5 ft. away from and 3 ft. above an infected ferret *must* have been due to particles below a given size. At any rate the higher level of a recipient's cage did not apparently interfere with the likelihood of cross-infection.

TABLE I.—*Infection of Ferrets across a Cubicle.*

Experiment.	No. of "donors."	Distance between ferrets.		Influenza "take" in recipients.	Incubation period in days.
		Total.	Vertical.		
1	2	5 ft.	Recipient 2 ft. 10 in. above donor	+	5
		2 ft. 3 in.	Level	+	5
2	1	2 ft. 3 in.	Level	+	11
		5 ft. 6 in.	Recipient 2 ft. 10 in. above donor	+	8
3	1	3 ft. 3 in.	Recipient 3 ft. above donor	+	7
		5 ft.	Recipient 3 ft. 6 in. above donor	0	–
4	2	,,	3 ft.	+	5
				+	8
5	2	,,	,,	+	3
				+	3
6	2	,,	,,	+	4
				+	4
7	1	,,	,,	0	–
8	2	,,	,,	+	6
				0	–
9	2; only one "took"	,,	,,	0	–
				0	–
10	2	,,	,,	? 0	–
				+	6
11	2	,,	,,	0	–
				0	–

Experiments with Screens.

In order to test whether infection was caused by droplets sneezed directly from one ferret to another, we interposed light plywood screens 4 ft. 6 in. by 2 ft. 6 in. in the centre of the cubicle so as to shield recipients from droplets directly projected by the infected ferrets. The screen had an opening at the base 1 ft. square, with its centre 1 ft. from the floor. A fan was fitted on the side nearest the recipient so that a current of air was blown towards the donor at a rate of about 20 ft. a second. It would then have to travel up past the donor and over the top of the screen in order to carry particles to the recipient. In two experiments, each with two donors and two recipients, no transmission of infection occurred, despite severe infection and repeated sneezing on the part of the donors. The success of our barrier against direct projection might be explained on the grounds that the coarser droplets are of major importance. Alternatively, one could argue that, since successful transmission did not occur in 100 per cent. of the control experiments, anything interfering with the numbers of infected particles in the air might lower the chances of success.

Experiments with Good Ventilation.

Tests on the rate of disappearance of CO_2 liberated into a cubicle showed that there was a very low air turnover when the doors and windows were shut. Increased ventilation of a cubicle might be expected to dilute the "infective" particles in the air and interfere with cross-infection, more especially, perhaps, if direct projectiles were not of prime importance. In two trials, each with two donors and two recipients, the windows in the cubicles were left wide open. The clean ferrets were placed near the bottom of the windows, and the current of air flowed from them towards the infected animals, then upwards and out of the window again at a higher level. In one of the trials the natural air currents were assisted by a fan placed near the donors. In neither experiment did any cross-infection occur, despite seven days' exposure of the normal ferrets to the infected ones.

Experiments with Ducts.

In order that factors, such as air currents, which could not be adequately controlled in a cubicle, might cease to act as possible sources of error, closed ducts were constructed of light wood, as shown in Fig. 1. The dimensions of the ducts as seen from above are indicated in the diagram; they were almost square in cross-section, $10\frac{1}{2}$ in. wide and $9\frac{1}{2}$ in. high, leaving room for small open-meshed wire cages 1 ft. 1 in. by $8\frac{1}{2}$ in. by 8 in. in height, one at each end. Access to the cages was secured by tightly fitting glass panels in the roof over each cage. The end-plate next to the donor ferret was perforated by 13 round holes, each 1 in. in diameter; the corresponding plate opposite the side of the recipient ferret's cage was similarly perforated, but with 13 holes, each $\frac{1}{4}$ in. in diameter. This arrangement was designed to ensure a uniform flow of air at a low speed in the same direction. Just outside the latter was a

four-blade electrically-driven extraction fan with a variable rheostat. As the figure shows, three ducts were built—straight, S-shaped and U-shaped respectively.

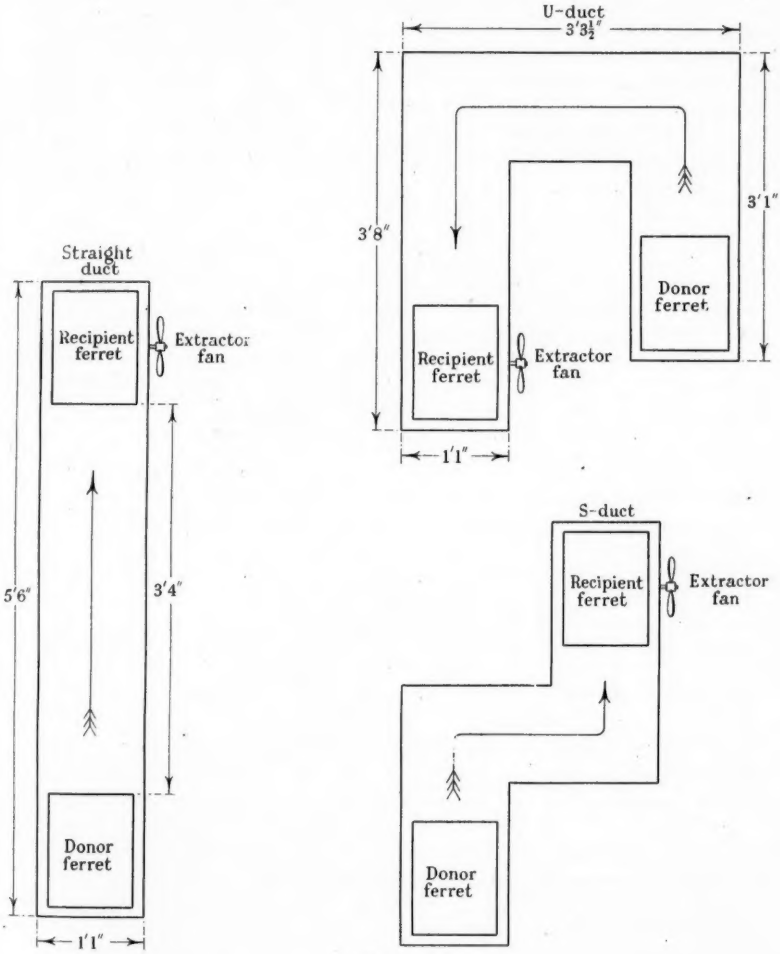

FIG. 1.

Two normal ferrets were first infected to act as donors. When fever and symptoms were in evidence after 48 hours they were placed, one each, in the donor cages of the straight and S-ducts and normal ferrets were put into the recipient cages. The fans were adjusted to draw through 14 linear feet of air a minute. Temperatures on the recipients were taken twice daily, particular

care being taken to avoid accidental contamination. Thus, before the ferrets were handled for any purpose, the fans were stopped and the animal concerned with its cage removed from the cubicle. In two further experiments (Exp. 13 and 14) the U-shaped duct was compared with the straight duct. The results are shown in Table II.

TABLE II.—*Experiments with Ducts.*

Experiment No.	No. of recipient ferret.	Duct used.	ft./min. Air-speed	Result.	Incubation period in days.
12	129	Straight	14	+	6 (?)
	130	S—	14	+	3
13	159	Straight	3	Mild take, immune later	5
	160	U—	3	Do.	7
14	177	Straight	13	+	8
	178	U—	13	+	6

From Exp. 12 it appeared that infection had occurred through the S-duct. It was thought likely that at the low air-speed employed coarse particles would not be swept round the bends, but would impinge upon the walls and be trapped. We soon perceived, however, that a U-shaped duct would test the point at issue more satisfactorily. As Table II shows, infection occurred round the bend of the U, both at the low and very low air-speeds. It seems highly improbable that droplets of the order of 0·1 to 1 mm. in diameter could be carried round corners at such low speeds, and the inference seems natural that infection was conveyed either by droplet-nuclei or very fine dust particles.

DISCUSSION.

There are several indications from the above experiments that the infection-bearing particles in influenza A of ferrets are not exclusively the gross droplets which are expelled when the animal sneezes. Infection occurs over a distance of over 5 ft. in almost still air; it can even travel upwards and infect a normal ferret in a cage several feet above an infected animal. Good ventilation, which would be expected to decrease the numbers of infective particles, seems to interfere with the chances of infection. Trials in ducts indicate that the particles are small enough to be wafted round right-angle bends by air-currents as sluggish as 3 ft. a minute. On the other hand, the importance of direct projectiles is suggested by the fact that screens interposed to shield recipient ferrets from direct droplets did seem to have value in stopping cross-infection. Unfortunately in control experiments infection across a cubicle did not take place with complete regularity; we cannot afford to lay as much weight on negative evidence, such as that provided by the screen and ventilation experiments, as on the positive results of others and of the duct trials.

Our experiments do not deny that coarse droplets may convey infection;

but they do suggest that very fine particles may also avail to transport influenza virus A. It is hoped to study later whether such fine particles are to be regarded as droplet nuclei in Wells's sense, or as minute fragments of dust.

It is difficult to study the mechanism of cross-infection when the infecting agent will not pass from one animal to another with perfect regularity. It was very encouraging, therefore, when one of our influenza-infected ferrets developed an added streptococcal infection, and thus provided us with a far more reliable method of attacking this problem ; experiments on the combined influenza A and streptococcus infection are described in following papers.

SUMMARY.

Influenza A infection will pass from one ferret to another across a distance of 5 ft. Raising the recipient ferret's cage to a level of 3 ft. above that of the infected animal does not prevent cross-infection. Increased ventilation or the interposition of a screen between the ferrets appear to decrease the chances of cross-infection ; but reasons are given for accepting the results of these tests with reserve. Trials in closed ducts suggest that infection may be conveyed by " droplet nuclei " or other very small particles.

We are very grateful to our colleagues, Drs. R. B. Bourdillon and O. M Lidwell, for designing and making the ducts used in the experiments.

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SPREAD OF INFECTION FROM THE RESPIRATORY TRACT OF THE FERRET. II. ASSOCIATION OF INFLUENZA A VIRUS AND STREPTOCOCCUS GROUP C.

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THE investigations on aerial transmission of influenza A infection in ferrets reported in the foregoing paper were handicapped by difficulties in controlling quantitative factors. A more fruitful field for study was opened by the chance occurrence of a streptococcal infection in a ferret inoculated with influenza virus. As will be shown, ferrets doubly infected with influenza A virus and streptococci regularly shed large numbers of cocci into the air; the numbers so emitted can be estimated. Experiments were put in hand with the object of studying, and, if possible, controlling the transfer of streptococci through the air; these will be described in subsequent papers. It is first desirable to give some account of the combined infection itself.

Isolation of Streptococcus (Group C) from the Ferret.

During the course of serial transfers of the W.S. strain of influenza virus A through ferrets, an animal was encountered which showed an unusually severe illness; the temperature rose to 106° F. 24 hours after inoculation, but by the following day had dropped to 98° F. The ferret then showed acute respiratory distress and was found dead later on the same day. Post-mortem examination revealed an intense haemorrhagic reaction of the nasal mucosa and a plum-coloured congestion involving nearly the whole of both lungs. Cultures from the turbinates gave a pure growth of an organism which proved to be a haemolytic streptococcus of Lancefield's Group C. Infected turbinates from a ferret sub-inoculated from this animal were dried *in vacuo* over P_2O_5 and afforded a source of material for subsequent experiments. It seems probable that streptococci present in the respiratory tract of one of the ferrets in the transmission series were stirred to activity by the influenza virus infection and carried on by the subsequent passage.

Character of the double infection in ferrets.—Dried turbinate obtained from the first passage ferret was used to infect six fresh animals by the intranasal route, at intervals ranging from 64 days up to 119 days after the material had been desiccated. In each instance a characteristic febrile reaction was obtained, quite dissimilar from that of uncomplicated influenza, in which a primary rise after an incubation period of approximately 48 hours is followed by a quiescent stage lasting one to three days, and at times, by a second exacerbation of varying duration. In the streptococcus-infected ferrets the temperature ranged from 104·6° F. to 106·6° F. between the 24th and 48th

hours, and remained at a high level for several days, varying from five to eight. If the animal survived there was some decline in the febrile response, but the temperature tended to fluctuate for at least ten days after infection. The differences in the temperature curves are shown in Figs. 1 and 2.

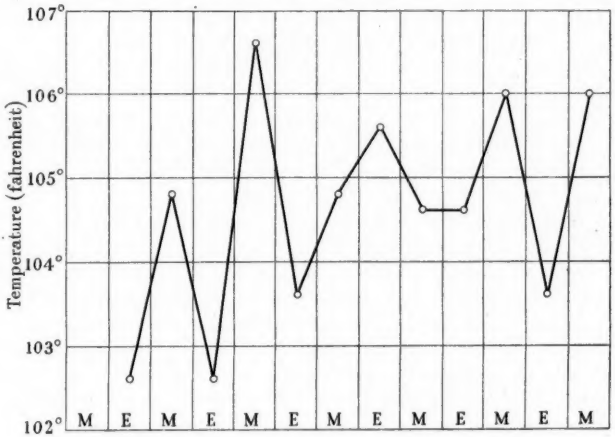

FIG. 1.—Ferret infected with influenza A virus + streptococcus.

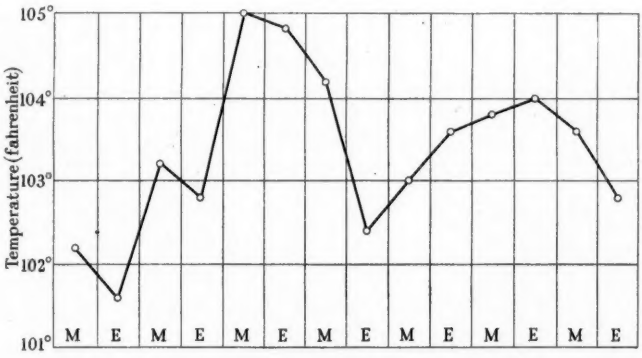

FIG. 2.—Ferret infected with influenza A virus only.

In the early stages the clinical symptoms were identical with those described by Smith, Andrewes and Laidlaw (1933) as occurring in ferrets infected with influenza virus, i.e. sneezing, yawning, nasal discharge and obstruction resulting from inflammation of the turbinates and nasal sinuses, but after the fourth day the nasal discharge became very profuse and consisted of a viscid purulent exudate, which tended to congeal round the external nares to such an extent that in many of the ferrets nasal breathing was completely

arrested. Animals in this stage were unable to free their nasal passages by sneezing and frequently attempted to remove the exudate with their paws.

In this series of six ferrets, one was killed at an early stage in the investigation, one died showing nearly complete consolidation of both lungs, while four survived although they were severely affected.

In addition to dried turbinate from the original ferret, the disease was set up by combining influenza virus with cultures of the haemolytic streptococcus. The virus, usually in the form of dried turbinate of an influenza-infected ferret, was suspended in broth saline and centrifuged to remove coarse particles, and added to an equal amount of a 24-hour broth culture of the streptococcus. The mixture (1 c.c.) was then instilled intranasally into ferrets without anaesthetic. Ten animals have been infected by this method, and in each case have shown febrile reactions and symptoms similar in nature to those following the inoculation of the dried turbinate containing the organism. The mortality in this series has been much higher, since five of the animals have died or have been killed when moribund. It was noticeable, however, that all the deaths occurred in the early part of the series—a fact which suggests that the streptococcus later became less virulent. Streptococci were recovered, usually in pure culture, from the turbinates of each animal, but only once from the lungs.

The organism alone in the absence of influenza virus proved incapable of establishing itself in the nose of the ferret. Two animals received 1 c.c. of a 24-hour culture by the intranasal route. The temperatures remained normal, no characteristic symptoms were detected, while nasal swabs taken each day were negative for haemolytic colonies.

Recovery of Streptococcus from Infected Ferrets.

The presence of the streptococcus in the nasal mucous membrane has been established by the recovery of the organism from the mucous or purulent secretions and by an examination of sections of turbinate.

It has been found that the simplest manner of isolating the organism from the nasal cavities is to use a stout Pasteur pipette, the end of which has been heated to form a blunt bulb. If necessary the external nares are freed from secretion by means of sterile wool swabs. The end of the pipette is then gently passed into each nasal passage, withdrawn and smeared over a blood-agar plate.

When the streptococcal culture is instilled simultaneously with influenza virus there is a latent period lasting about 48 hours when practically no colonies are observed ; this period corresponds with the pre-febrile stage. As soon as the first temperature reaction is well established the organism can be recovered, at first in small numbers, but later with increasing frequency. From the fourth day over a period of five to six days the streptococci occur in very large numbers, but thenceforward they steadily diminish while other organisms, particularly Gram-negative bacilli of the proteus group, tend to complicate the picture. It is not necessary, however, to introduce the streptococcal culture at the same time as the virus in order to enable the former to become established in the nasal sinuses, since infection is readily secured if an interval is allowed between the two inoculations.

In a typical experiment a ferret was infected by the intranasal route with a mixture of PR8 strain of virus and a culture of streptococcus. At the

TABLE I.—*Infection of Ferret with Streptococcus after PR8 Virus.*

No. of ferret.	Date of infection with PR8 virus.	Date of infection with streptococcus.	Interval.	Streptococcal count.				
				4/11.	5/11.	7/11.	9/11.	12/11.
217	23/10/40	23/10/40	0	±	C	..	..	..
209	,,	26/10/40	3	++	++	±	±	—
200	,,	27/10/40	4	++	++	++	+	±
201	,,	28/10/40	5	++	++	+	C	..
199	,,	30/10/40	7	++	±	—	—	..

++ Heavy growth.
\+ Moderate growth.
± Slight growth.
C Overgrown with other organisms.
— No growth.

same time four more ferrets received PR8 virus only, and were subsequently infected in turn with the streptococcus at intervals of 3, 4, 5 and 7 days respectively. The presence of the streptococci in the nasal mucous membrane was demonstrated by daily examinations of the secretions until such time as they had completely disappeared or had become so masked with other organisms that they were no longer detectable. The results are depicted in Table I.

From these few experiments it would seem that streptococci are just as likely to persist when administration of the organism is deferred to the third or fourth day after the virus, as when the two agents are introduced simultaneously.

Aerial Spread of Streptococcus Group C from Infected Ferrets.

The experiments on the recovery of streptococci from the air have been conducted in the small cubicles of the ferret isolation hospital. In order that the result of any one experiment might be compared with another, the conditions under which the ferrets have been maintained have been kept as constant as possible, although minor variations have been introduced as the investigation progressed.

In most instances the donor ferrets have been infected by the simultaneous nasal instillation of ferret adapted influenza virus (WS or PR8 strains) and a broth culture of the group "C" streptococcus, but in a few recent experiments the ferret has received influenza virus first and the streptococcus as soon as the febrile reaction has commenced. The nasal secretion was plated every 24 hours until an abundant growth of the organism was obtained. The animal was then transferred to a cubicle measuring 6 ft. 9 in. by 4 ft. 9 in., the window of which was darkened to avoid the effect of light on the bacteria. The ferret was housed in a wire mesh cage without litter, and supported 1 ft. 6 in. from the ground in one corner of the room. Blood-agar plates were then exposed in six different positions, viz., 2 in. in front of the cage (*a*), on a ledge 3 ft. above the cage (*b*), and on two shelves (*c*, *d*, *e*, *f*), one level with the ferret and the other 2 ft. above it. On the higher shelf the plates were placed in positions at a horizontal distance of 1 ft. (*c*), and 4 ft. from the ferret (*e*), with corresponding positions (*d*) and (*f*) on the lower shelf.

Streptococci were recovered in all these positions, the numbers naturally showing some variation according to the distance from the ferret, the height from the floor, and the time of exposure. Table II shows the results of three successive experiments; in the first the plates were exposed for three hours, in the second duplicate plates were placed in each position, one of each pair being opened for one hour and the other for three hours, while in the third there were three plates, each one being opened for one hour.

It is noticeable that the greatest number of organisms always appeared on the plates nearest to the ferret, and that the lower plates showed more colonies than the upper.

Evidence that influenza A virus can be transmitted over a distance of several feet has already been presented (Andrewes and Glover, 1941). The effect of the association of influenza virus and the haemolytic streptococcus on the aerial spread of each component has also been studied. Two normal ferrets in separate rabbit cages were exposed in a cubicle with two ferrets which had received a mixture of WS virus and a culture of streptococcus by the intranasal route four days previously. The donor animals were placed in open mesh wire cages 1 ft. 6 in. above floor level and were separated by a space of about 6 ft. from the recipients, which were located at a height of

TABLE II.—*Three Successive Plate Exposures.*

Position of plate.	Plates exposed 3 hours.	Plate (1) 1 hour. Plate (2) 3 hours.	Plate (1) 1st hour. Plate (2) 2nd hour. Plate (3) 3rd hour.
a	141	(1) 77	(1) 43
		(2) 128	(2) 56
			(3) 32*
b	117	(1) 10	(1) 27
		(2) 28	(2) 35
			(3) 10
c	92	(1) 6	(1) 12
		(2) 10	(2) 17
			(3) 4
d	134	(1) 5	(1) 15
		(2) 21	(2) 18
			(3) 3
e	78	(1) 8	(1) 13
		(2) 24	(2) 12
			(3) 5
f	113	(1) 5	(1) 15
		(2) 25	(2) 17
			(3) 2

	Distance of plates from ferret.	
	Horizontal.	Vertical (above).
a	2 in.	0 in.
b	1 ft.	3 ft.
c	1 ,,	2 ,,
d	1 ,,	0 ,,
e	4 ,,	2 ,,
f	4 ,,	0 ,,

* During this period ferret was asleep.

4 ft. 6 in. from the floor. At the same time the experiment was duplicated in another cubicle, except that the donor ferrets received influenza virus only. In the case of the double infection, both recipients failed to show any temperature response or clinical symptoms of influenza, whereas both the ferrets exposed to the uncomplicated virus infection went down at the sixth and seventh days respectively. A repetition of the experiment gave an identical answer. In each instance numerous streptococci were recovered from the nasal secretions of the doubly infected ferrets, while cultures from the recipients were negative.

The reason for the failure of the two agents combined to infect the normal ferret is not obvious, but a possible explanation is to be found on physical grounds. It was observed that after the fourth or fifth day the nasal orifice tended to become completely blocked with a thick exudate, forming a crust which was so sticky that animals often could not breathe through the nose.

Such ferrets made frequent unsuccessful attempts to clear their nasal passages by sneezing. It might be assumed, therefore, that there was a mechanical barrier to the extrusion of a sufficient number of influenza virus

particles into the surrounding air to infect the normal ferret. A certain number of streptococci escaped, since plates exposed at the side of the cages containing the recipient ferrets showed small numbers of colonies. Confirmation was thus obtained of previous experiments, indicating that in the absence of influenza virus streptococci are unable to become established in the nasal mucous membrane.

The role of the virus in facilitating the entry of bacteria was also studied in a different manner both by immediate and air-borne contact experiments.

(a) *Immediate contact.*—In these experiments a normal ferret was placed in the same metal cage as an animal which had received a mixture of WS virus and streptococcus three days previously and was showing numerous streptococci in the nasal secretions ; 24 hours later the ferrets were separated. The normal ferret showed a sharp febrile response within 48 hours of isolation and cultures from its nose were positive. At the seventh day when the original ferret was still spreading streptococci a normal animal was again put in contact with it, left for 24 hours and then removed. In this case, however, the ferret failed to show a temperature reaction and no streptococci were recovered from its nose.

We knew from our earlier experiments, lately confirmed by those of Horsfall and Lennette (1940), that influenza virus is not recoverable from the turbinates at the sixth day after inoculation. In order to determine whether the virus had actually disappeared from doubly infected ferrets, an animal inoculated seven days previously was killed and a gradocol filtrate (770m.μ) was prepared from the turbinates and lungs. When this was introduced intranasally into a fresh ferret no temperature reaction followed, and there were no clinical symptoms.

(b) *Air-borne contact.*—If the assumption is correct that the establishment of the streptococcus is dependent upon the preparation of the mucous membrane by some other agent such as influenza virus, it should be possible to provide the necessary conditions by exposing ferrets suffering from pure influenza virus infection to the streptococci emitted by a doubly infected ferret in the same cubicle. Such proved to be the case.

Ferret 204, infected with WS virus plus streptococcus and placed in a rabbit cage when it was shedding numerous cocci into the air, was separated by the length of a cubicle from two ferrets, 203 and 205, each in separate cages. F.203 had received a nasal instillation of PR8 48 hours previously, while F.205 was a normal animal. Twenty-four hours later the three ferrets were isolated in separate cubicles and cultures of their nasal secretions were made daily on blood-agar plates. F.203 showed very numerous haemolytic streptococci within 24 hours of removal from the cubicle, while F.205 was negative up to the 7th day ; it was subsequently shown to be susceptible to influenza, whereas F.203 was immune. The failure of F.205 to contract influenza from F.203 was probably due to the short time the two animals were in contact.

Infection of Tonsils.

In view of the importance of the tonsils as a focus in human streptococcal infections, this site was examined in a number of ferrets in the latter part of

TABLE III.—*Recovery of Streptococcus from Ferret.*

No. of ferret.	Interval between virus and streptococcus. (Days.)	Interval to P.M. (Days.)	Plates in cubicle.	Nasal secretion.	Turbinate.	Tonsil.	Trachea.	Lung.
233	5	1	+++	+++	++++	++++	+	—
228	5	9	+	+	++	++	+	—
226	5	12	—	—	—	+++	0	—
227	4	14	—	—	—	+++	—	—
225	0*	29	—	—	—	—	0	—

++++ Very heavy growth.
+++ Heavy growth.
++ Moderate growth.
\+ A few colonies.
— No colonies.
0 Not done.

* Simultaneous.

the series. The first ferret which was selected had been emitting streptococci into the air for nine days, but plates exposed on the 10th and 11th days were negative. At post-mortem the nasal mucosa was covered with a thin, tenacious film, while the turbinates were very slightly muco-purulent. Both tonsils were enlarged to about one and a half times the normal size. Cultures from the nose, turbinates and lungs were free from streptococci, but both tonsils yielded numerous colonies of streptococci in almost pure culture. In all, five ferrets have been examined with particular reference to the localization of streptococci in the tonsils (Table III). In each case blood-agar plates were exposed in a cubicle with the ferret immediately before autopsy. In two ferrets (233 and 228), in which the streptococci had been instilled one day and nine days previously, the plates were positive, and in these instances haemolytic colonies were also obtained from nasal secretion, turbinate, tonsil and upper trachea (small numbers only) but not from the lungs. Two ferrets (226 and 227) infected 12 and 14 days beforehand respectively were not emitting streptococci into the air, and were free from the organism in so far as the nasal secretion, turbinate and trachea were concerned, but the tonsils were positive. By the 29th day all sites were completely negative (225).

It is interesting that the failure to recover organisms from the air in the cubicle ran parallel with their disappearance from the nasal secretion and turbinate, whereas the streptococci persisted in the tonsils for a longer period. It has already been noted that streptococcus administered intranasally in the normal ferret does not establish itself in the nose. It has been found, however, that the organism can become localized in the tonsils. F.142, which received a broth culture, was tested for air-borne streptococci three and four days after infection with negative results. At post-mortem on the fourth day, cultures from the nasal mucous membrane and turbinates were negative, but abundant streptococci in almost pure culture were recovered from both tonsils.

DISCUSSION.

The association of viruses with bacteria in certain respiratory diseases is universally recognized, but the possible influence of the former on the aerial spread of organisms is less well understood. The lack of a suitable small animal convertible into a carrier of a selected organism, which is not only readily shed into the air, but is also easily identified, has undoubtedly hindered laboratory studies of the methods whereby bacteria are expelled from the upper respiratory passages. The opportunity presented by the concurrent infection of ferrets with influenza A virus and a haemolytic streptococcus group C has opened a new method of approach which will probably yield some interesting results.

It was found that during the early stages of the double infection comparatively large numbers of the streptococci were emitted, although the numbers of the colonies recovered on blood-agar plates exposed at varying distances from the ferret fluctuated from hour to hour. Swabs showed that these nasal secretions were very rich in streptococci, often in nearly pure culture, and it is tempting to suppose that the particles detected in the cubicles were expelled when the animal sneezed. Undoubtedly this did occur, but the

possibility that the organisms were shed by other channels must not be overlooked. It is of interest that the tonsils were infected as soon as the nasal mucosa and that the streptococci tended to persist in the former for a longer period. It is uncertain whether in the early stages air-borne streptococci were derived from the nasal secretion alone, or whether tonsillar infection also played a part. It is significant, however, that the emission of the streptococci into the air ceased as soon as the turbinates and nasal cavities had freed themselves of the organisms.

It is an interesting point that the establishment of the streptococci is so closely linked with the active stage of influenza infection. In the absence of the virus the organism is unable to gain a footing in the nose, but once a breach has been effected it persists in the secretions for a relatively long period. The inability of the organism to infect the nasal tissues of the normal ferret seems to imply that damage to the cells is the predisposing factor. Apparently, however, the nature of such damage is not a matter of indifference, since the application of a zinc sulphate solution according to the method of Stuart-Harris and Francis (1938) failed to reproduce the conditions necessary for the localization of the streptococci instilled a few hours later.

CONCLUSIONS.

(1) A nasal infection of the ferret has been induced by the combined action of influenza A virus and a haemolytic streptococcus (group C).

(2) The disease was evoked by dried ferret turbinates containing both agents, or by a mixture of virus and broth culture of the organism. The administration of the streptococcus up to seven days after the virus was effective.

(3) The streptococcus was recovered from the nasal mucosa, the turbinates and tonsils. It was incapable of infecting the nasal passages in the absence of influenza virus.

(4) The streptococcus invariably established itself in the noses of clean ferrets placed in close contact with doubly-infected ferrets. When the animals were separated by an air barrier of 4 ft. the streptococci failed to infect the nose of normal ferrets, but did infect animals suffering from influenza A.

(5) The organisms shed into the air by carriers were readily recovered by the exposure of blood-agar plates. This fact has made possible the direct experimental study of air-borne transmission of infection, as will be reported in subsequent communications.

ACKNOWLEDGMENT.

Especial thanks are due to Dr. C. H. Andrewes, F.R.S., whose helpful advice has been most valuable.

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TYRAMINASE ACTIVITY IN THE HYPERTENSIVE ANIMAL.

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IT has been shown that the pressor response to adrenaline, tyramine, and posterior pituitary extract is increased in the hypertensive animal (Brown and Maegraith, 1941). A possible cause of this phenomenon is a decreased rate of enzymatic destruction of pressor substances. The validity of this suggestion has been tested by determining the rate of oxidation of tyramine in tissue from normal and from hypertensive animals.

METHODS.

The experiments were carried out on rats.

Blood pressure.—The blood pressure was estimated by a modification of the method of Moberg (1934), the animal being anaesthetized with nembutal. The normal blood pressure for 72 rats varied from 41 to 58 mm. Hg, with an average of 50 mm. Hg. In hypertensive rats, pressures up to 100 mm. Hg were obtained. The blood pressure as estimated by this method proved to be independent of variation in the dose of nembutal and of variation in room temperature from 15° to 27°C. Moberg (1934) found the normal range of a small series of animals to be 45 to 75 mm. Hg. Smadel and Farr (1937) using the same method found it to be 40 to 60 mm. Hg.

Constriction of the renal artery.—The renal artery was constricted with a small Perspex clamp. It consisted of a block of the material measuring 4 mm. by 2 mm. by 1 mm., and having cut into it a slot 0·4 mm. wide and 3 mm. deep. The clamp was held in place on the artery by a thread tied into a groove around its open end. This method of constriction resulted in an increase of blood pressure in 75 per cent. cases. Bilateral constriction, or constriction of the artery to the remaining kidney after unilateral nephrec tomy, caused a permanent elevation of blood pressure. Unilateral constriction in rats fed on a diet of oats, bread and biscuit caused only a temporary hypertension.

Estimation of tyraminase activity.—The usual Warburg manometric technique was employed. The experiments were done in triplicate with liver slices in an atmosphere of oxygen at 37° C. M/15 phosphate buffer, pH 7·4, was used throughout, and determinations were made at two concentrations of tyramine, 0·0025 *M* and 0·0125 *M*. The saturating concentration was found to be 0·005 *M*. Q_{O_2} was estimated as

$$\frac{\text{cubic millimetres of oxygen consumed}}{\text{hours} \times \text{milligramme tissue (dry weight)}}$$

Preliminary experiments showed that the use of slices cut from frozen tissue would simplify procedure. Freezing in the refrigerator reduced the residual oxygen consumption to almost zero, but left unaffected the initial Q_{O_2} following

the addition of tyramine. Ammonia estimations by the method of Conway (1933) showed that the rate of deamination was the same in the two cases. It was possible, therefore, to use the mean Q_{O_2} during the first 20 minutes of the reaction with previously frozen tissue as the measure of tyraminase activity.

RESULTS.

The tyraminase activity in a 0·0025 *M* solution of tyramine was determined for 5 hypertensive and 13 normal rats. The results are shown in Table I.

TABLE I.—*Comparison of Activity of Tyraminase of Livers of Normal and Hypertensive Rats.* 0·0025 M *Tyramine.*

Normal rats.		Hypertensive rats.		
No.	Q_{O_2}.	No.	B.P. (mm. Hg.).	Q_{O_2}.
1739	13·0	1170	90	15·1
1740	12·7	1214	65	12·9
1741	13·0	1222	72	14·0
1742	15·0	1225	74	12·9
1743	15·2	1231	77	13·3
1744	15·4			
1745	13·7			
1746	13·2			
1747	15·2			
1748	13·5			
1749	11·5			
1750	14·9			
1751	14·2			
Average 13·9 ± 1·06 s.d.		Average 13·7 ± 2·64 s.d.		

TABLE II.—*Comparison of Activity of Tyraminase of Livers of Normal and Hypertensive Rats.* 0·0125 M. *Tyramine.*

Normal rats.		Hypertensive rats.		
No.	Q_{O_2}.	No.	B.P. (mm. Hg.).	Q_{O_2}.
1790	15·8	1170	90	28·4
1791	19·6	1214	65	19·1
1792	20·3	1216	67	14·8
1793	30·4	1217	87	19·8
1794	21·0	1222	72	21·0
1795	20·8	1225	74	24·7
1796	25·3	1229	70	26·0
1797	28·4	1231	77	20·6
1798	23·8	1722	75	16·7
1799	16·1	1724	80	25·2
1800	13·5	1726	80	18·8
1801	17·2	1731	68	20·0
1802	15·9			
1803	20·2			
1804	13·4			
Average 20·1 ± 4·1 s.d.		Average 21·2 ± 3·4 s.d.		

The activity in 0·0125 *M* solution was determined for 12 hypertensive and 15 normal rats. These results are given in Table II. In neither series is there any deviation from normal. The hypertensive rats had shown elevated blood pressures for periods of 4 weeks to 15 months.

DISCUSSION.

It is evident that, in so far as it has been tested *in vitro*, the activity of the tyraminase of the liver of the hypertensive animal is the same as that found in the normal animal both at a concentration of the substrate which ensures saturation of the enzyme and at a concentration which reveals its activity when it is but partly saturated. The conditions of the experiment are very different from those present *in vivo*, but the results do not support the suggestion that the rate of oxidation of tyramine is reduced in hypertension.

Other considerations argue against this possibility. If the altered responses to adrenaline, tyramine, posterior pituitary extract and acetyl choline which were demonstrated by Brown and Maegraith (1941) are to be explained on a basis of reduced enzymatic activity, there must be at least three enzyme systems involved. This seems unlikely. Also, in the hypertensive animal there is no proportional increase in the duration of the pressor response to adrenaline, such as was seen by Frölich and Loewi (1910) following cocainization, nor an increase in the duration of the pressor response to either adrenaline or tyramine similar to that which was seen by Tripod (1940) in animals previously injected with butyn.

Conclusion.—There is no evidence that the increased sensitivity to pressor substances demonstrated in the hypertensive animal is due to a diminished rate of enzymatic destruction of these substances.

SUMMARY.

The activity of tyraminase in the liver of the hypertensive rat has been tested *in vitro* and found to be normal.

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